

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
18 March 2004 (18.03.2004)

PCT

(10) International Publication Number  
**WO 2004/022593 A2**

- (51) International Patent Classification<sup>7</sup>: **C07K 14/52** 49 rue Félix Faure, F-91270 VIGNEUX-SUR-SEINE (FR).
- (21) International Application Number: PCT/IB2003/004347 (74) Agent: CABINET ORES; 36 rue de St Pétersbourg, F-75008 PARIS (FR).
- (22) International Filing Date: 8 September 2003 (08.09.2003) (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
60/409,898 9 September 2002 (09.09.2002) US  
60/457,135 21 March 2003 (21.03.2003) US
- (71) Applicant (*for all designated States except US*): NAUTILUS BIOTECH [FR/FR]; 1 rue Pierre Fontaine, F-91000 EVRY (FR).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): GANTIER, René [FR/FR]; 2 rue de Dublin, La Clef de Saint-Pierre, F-78990 ELANCOURT (FR). GUYON, Thierry [FR/FR]; 8 résidence les Terres Rouges, F-91120 PALAISEAU (FR). VEGA, Manuel [IT/FR]; 49 rue Félix Faure, F-91270 VIGNEUX-SUR-SEINE (FR). DRITTANTI, Lila [IT/FR];
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**  
— without international search report and to be republished upon receipt of that report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: RATIONAL EVOLUTION OF CYTOKINES FOR HIGHER STABILITY, THE CYTOKINES AND ENCODING NUCLEIC ACID MOLECULES

(57) Abstract: Processes and systems for the high throughput directed evolution of peptides and proteins, particularly cytokines that act in complex biological settings, are provided. Also provided is a rational method for generating protein variants and the resulting variants.

WO 2004/022593 A2

BEST AVAILABLE COPY

-1-

**RATIONAL EVOLUTION OF CYTOKINES FOR HIGHER STABILITY, THE  
CYTOKINES AND ENCODING NUCLEIC ACID MOLECULES**

**RELATED APPLICATIONS**

Benefit of priority to U.S. Provisional Application Serial No.

- 5 60/457,135, entitled "RATIONAL EVOLUTION OF CYTOKINES FOR  
HIGHER STABILITY, ENCODING NUCLEIC ACID MOLECULES AND  
RELATED APPLICATIONS," filed March 21, 2003, and to U.S. Provisional  
Application Serial No. 60/409,898, entitled "RATIONAL EVOLUTION OF  
CYTOKINES FOR HIGHER STABILITY, ENCODING NUCLEIC ACID  
10 MOLECULES AND RELATED APPLICATIONS," filed September 9, 2002,  
each to Rene Gantier, Thierry Guyon, Manuel Vega and Lila Drittanti.

- This application is related to U.S. application Serial No. attorney  
dkt. no. 37851-922, entitled, "RATIONAL EVOLUTION OF CYTOKINES  
FOR HIGHER STABILITY, THE CYTOKINES AND ENCODING NUCLEIC  
15 ACID MOLECULES," to Rene Gantier, Thierry Guyon, Manuel Vega and  
Lila Drittanti. This application also is related to U.S. application Serial No.  
Attorney docket no. 37851-923, filed the same day herewith, entitled  
"RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL  
RATIONAL MUTAGENESIS SCANNING," to U.S. provisional application  
20 Serial No. 60/457,063, entitled "RATIONAL DIRECTED PROTEIN  
EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS  
SCANNING," filed March 21, 2003, and to U.S. Provisional Application  
Serial No. 60/410,258, entitled "RATIONAL DIRECTED PROTEIN  
EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS  
25 SCANNING," filed September 9, 2002, each to Rene Gantier, Thierry  
Guyon, Hugo Cruz Ramos, Manuel Vega and Lila Drittanti. This  
application also is related to co-pending U.S. application Serial No.  
10/022,249, filed December 17, 2001, entitled "HIGH THROUGHPUT

-2-

DIRECTED EVOLUTION BY RATIONAL MUTAGENESIS," to Manuel Vega and Lila Drittanti.

Where permitted, the subject matter of each of the above-noted applications and provisional applications is incorporated by reference in its  
5 entirety.

#### FIELD OF INVENTION

Modified cytokine proteins having selected modified properties compared to the unmodified or wild type proteins, and nucleic acid molecules encoding these proteins are provided. Uses of these proteins  
10 for diagnosis and treatment of diseases also are provided.

#### BACKGROUND

The delivery of therapeutic proteins for clinical use is a major challenge to pharmaceutical science. Once in the blood stream, these proteins are constantly eliminated from circulation within a short time by  
15 different physiological processes, involving metabolism as well as clearance using normal pathways for protein elimination, such as (glomerular) filtration in the kidneys or proteolysis in blood. The latter is often the limiting process affecting the half-life of proteins used as therapeutic agents in per-oral administration and either intravenous or  
20 intramuscular injection. The problems associated with these routes of administration of proteins are well known and various strategies have been used in attempts to solve them.

A protein family, which has been the focus of much clinical work, and efforts to improve its administration and bio-assimilation, is the  
25 cytokine family, including the interferon family. Interferon molecules are grouped in the heterogeneous family of cytokines, originally identified on the basis of their ability to induce cellular resistance to viral infections (Díaz *et al.*, *J. Interferon Cytokine Res.*, 16:179-180, 1996). Type I interferons, referred to as interferons  $\alpha/\beta$ , include many members of the

-3-

interferon  $\alpha$  family (interferon  $\alpha 1$ ,  $\alpha 2$ ,  $\omega$  and  $\tau$ ) as well as interferon  $\beta$ .

The type II interferon  $\gamma$  is different from type I in its particular mechanisms that regulate its production. Whereas the production of interferons  $\alpha/\beta$  is most efficiently induced in many types of cells upon

5 viral infection, interferon- $\gamma$  is produced mainly in cells of hemopoietic system, such as T-cells or natural killer cells, upon stimulation by antigens or cytokines, respectively. These two interferon systems are functionally non-redundant in the antiviral defense host.

Interferon  $\alpha$ , hereinafter "interferon alpha-2b," or "interferon  $\alpha$ -2b" or "IFN $\alpha$ -2b," used interchangeably, has a broad spectrum of biological effects, including antiviral effects. Antiviral effects include antiproliferative and immuno-modulatory actions (Stark *et al.*, *Annu. Rev. Biochem.*, 67:227-264, 1998). As well as eliciting strong antiviral activities in target cells, interferons  $\alpha/\beta$  also activate effector cells of the

15 innate immune system such as natural killer cells and macrophages (Pestka *et al.*, *Annu. Rev. Biochem.*, 56:727-777, 1987; Biron *et al.*, *Annu. Rev. Immunol.*, 17:189-220, 1999). As part of its immuno-modulatory action, interferon type I protects T-lymphocytes from apoptosis (Scheel-Toeller *et al.*, *Eur. J. Immunol.*, 29:2603-2612, 1999;

20 Marrack *et al.*, *J. Exp. Med.*, 189:521-530, 1999) and growth enhancing factors (Robert *et al.*, *Hematol. Oncol.*, 4:113-120, 1986; Morikawa *et al.*, *J. Immunol.*, 139:761-766, 1987). The biological effects of interferons  $\alpha/\beta$  are initiated upon binding to the IFN type I receptor, which results in activation of several downstream effector molecules (Hibbert

25 and Foster, *J. Interferon Cytokine Res.*, 19:309-318, 1999).

Interferons as well as many cytokines are important therapeutics. Since naturally occurring variants have not evolved as therapeutics, they often have undesirable side-effects as well as the above-noted problems of short-half life, administration and bioavailability. Hence, there is a need



-4-

to improve properties of cytokines, including interferons, for use as therapeutic agents. Therefore, among the objects herein, it is an object to provide cytokines that have improved therapeutic properties.

#### SUMMARY

5            Provided herein are methods for directed evolution of families of proteins and resulting families of modified proteins. A family, such as the cytokine protein family, is initially identified. A property or phenotype for modification, such as resistance to proteolysis for increased stability in blood, is selected for modification. A representative member or members  
10 of the family, such as members of the interfero  $\alpha$  family, such as IFN $\alpha$ -2b or IFN $\alpha$ -2a, or interferon  $\beta$  family, is (are) selected. It is modified using any directed evolution method and protein(s) with a desired phenotype are screened and identified. In addition, the 3-dimensional structure of the protein can be mapped to topologically and spatially identify the loci  
15 that are modified to achieve the phenotypic change. 3-dimensional structures of other members of the family are generated or obtained and compared with the modified family member. Loci in the other family members that correspond on the protein to those modified in the original protein are identified and modified. The resulting proteins can be tested  
20 to confirm that they exhibit the modified phenotype.

            Provided herein are methods for generating modified cytokines based on structural homology (3D scanning). These methods are based on the spatial and topological structure; they are not based on their underlying sequences of amino acid residues. The methods are used for  
25 identification of target sites for mutagenesis, particularly in families of target proteins. The targets are identified through comparison of patterns of protein backbone folding between and among structurally related

-5-

proteins. The methods are exemplified herein for cytokines. Families of the modified cytokines also are provided herein.

Any protein known or otherwise available to those of skill in the art is suitable for optimization using the directed evolution methods provided  
5 herein, including cytokines (e.g., IFN $\alpha$ , including IFN $\alpha$ -2b and IFN $\alpha$ -2a, and IFN $\beta$ ) or any other proteins that have already been mutated or optimized.

Provided herein are modified cytokines that exhibit increased resistance to proteolysis as assessed *in vivo* or *in vitro*. Typically the  
10 increase in resistance is at least 5%, generally 8%, 10% or more. The modified cytokines provided herein include those designed by 3D scanning using the interferon  $\alpha$ 's that were modified based upon 2D scanning methods herein.

Also provided herein are modified (mutant) cytokine proteins, such  
15 as variants of IFN $\beta$  and IFN $\alpha$ , including IFN $\alpha$ -2b and IFN $\alpha$ -2a proteins and IFN $\beta$  proteins, that have altered, particularly, improved therapeutic properties, including higher stability compared to the unmodified forms. In particular, exemplary modified cytokines provided herein have increased stability, which, for example, improves their use as  
20 therapeutics. Among the modified cytokines provided herein are those that exhibit increased resistance to proteolysis compared to the unmodified cytokine. In particular, such resistance is at least 10%, 20%, 30%, 40%, 50%, 70%, 100% or more resistant to proteolysis compared to the unmodified cytokine. Also provided are cytokines that have  
25 increased anti-proliferative and/or antiviral activity and/or resistance to proteolysis compared to an unmodified cytokine.

Exemplary of the modified cytokines provided herein are modified interferons that exhibit higher stability compared to unmodified forms. Such modified interferons can be used for treating conditions in humans

that are responsive to treatment with interferons, such, but are not limited to, as viral infections, cancer or tumors, undesired cell proliferation and for immuno-modulation.

Exemplary of proteins that can be modified by the 2D and 3D

- 5 scanning methods provided herein are cytokines from the interferons/interleukin-10 family. This family includes, for example, interleukin-10 (IL-10; SEQ ID NO:200, interferon beta (IFN $\beta$ ; SEQ ID NO: 196), interferon alpha-2a (IFN $\alpha$ -2a; SEQ ID NO: 182), interferon alpha-2b (IFN $\alpha$ -2b; SEQ ID NO:1), and interferon gamma (IFN- $\gamma$ ; SEQ ID NO: 199).
- 10 The long-chain cytokine protein family includes, among others, granulocyte colony stimulating factor (G-CSF; SEQ ID NO: 210), leukemia inhibitory factor (LIF; SEQ ID NO: 213), growth hormone (hGH; SEQ ID NO: 216), ciliary neurotrophic factor (CNTF; SEQ ID NO: 212), leptin (SEQ ID NO: 211), oncostatin M (SEQ ID NO: 214), interleukin-6 (IL-6; SEQ ID NO: 217) and interleukin-12 (IL-12; SEQ ID NO: 215). The short-chain cytokine protein family includes, among others, erythropoietin (EPO; SEQ ID NO: 201), granulocyte-macrophage colony stimulating factor (GM-CSF; SEQ ID NO: 202), interleukin-2 (IL-2; SEQ ID NO: 204), interleukin-3 (IL-3; SEQ ID NO: 205), interleukin-4 (IL-4; SEQ ID NO: 207), interleukin-5 (IL-5; SEQ ID NO: 208), interleukin-13 (IL-13; SEQ ID NO: 209), Flt3 ligand (SEQ ID NO: 203) and stem cell factor (SCF; SEQ ID NO: 206).
- 20 Modified forms of each that have increased resistance to proteolysis are provided. They were generated by comparison among the 3D-structures to identify residues that improve resistance to proteolysis.
- 25 Pharmaceutical compositions containing each modified cytokine and uses and methods of treatment are provided.

-7-

The modified cytokines have use as therapeutics. Each cytokine has improved biological and or therapeutic activity, compared to the know activity of the unmodified cytokine. Accordingly, uses of the cytokines for treatment of cytokine-mediated diseases and diseases for which immunotherapy is employed are provided. Methods of treatment using the modified cytokines for diseases also are provided. Each cytokine has a known therapeutic use, and such use is contemplated herein. Cytokines provided herein have improved properties, such as increased bioavailability, improved stability, particularly *in vivo*, and/or greater efficacy.

#### DESCRIPTION OF THE FIGURES

Figure 1(A) displays the sequence of the mature IFN $\alpha$ -2b. Residues targeted by a mixture of proteases, including  $\alpha$ -chymotrypsin (F, L, M, W, and Y), endoproteinase Arg-C (R), endoproteinase Asp-N (D), endoproteinase Glu-C (E), endoproteinase Lys-C (K), and trypsin (K, and R), are underlined and in bold lettering

Figure 1(B) displays the structure of IFN $\alpha$ -2b obtained from the NMR structure of IFN $\alpha$ -2a (PDB code 1ITF) in ribbon representation. Surface residues exposed to the action of the proteases considered in FIG1A are in space filling representation.

Figure 2 depicts the "Percent Accepted Mutation" (PAM250) matrix Values given to identical residues are shown in gray squares. Highest values in the matrix are shown in black squares and correspond to the highest occurrence of substitution between two residues.

Figure 3 presents the scores obtained from PAM250 analysis for the amino acid substitutions (replacing amino acids on the vertical axis; amino acid position on the horizontal axis) aimed at introducing resistance to proteolysis into the IFN $\alpha$ -2b at the protease target sequences. The two

-8-

best replacing residues for each target amino acid according to the highest substitution scores are shown in black rectangles.

Figures 4(A)-4(C) provide graphs of experiments indicating the levels of protection against *in vitro* proteolysis for IFN $\alpha$ -2b variants  
5 produced in mammalian cells. In Figures 4(B) and 4(C), the vertical axis indicates the relative level of non-proteolized protein and the horizontal axis indicates time in hours.

Figure 5 displays the characterization of several IFN $\alpha$ -2b variants, produced in mammalian cells, treated with  $\alpha$ -chymotrypsin.

10 Figure 6(A) shows the characterization of the E113H IFN $\alpha$ -2b variant when treated with  $\alpha$ -chymotrypsin. The percent of residual (anti-viral) activity for the variant (black line and white circles) after treatment with  $\alpha$ -chymotrypsin was compared to the treated wild-type IFN $\alpha$ -2b (dashed line and black squares). For this experiment, the E113H IFN $\alpha$ -2b  
15 variant was produced in mammalian cells.

Figure 6(B) shows the characterization of the E113H IFN $\alpha$ -2b variant treated with a mixture of proteases. The percent of residual (anti-viral) activity for the variant (black line and white circles) after treatment with protease mixture was compared to the treated wild-type IFN $\alpha$ -2b  
20 (dashed line and black squares). For this experiment, the E113H IFN $\alpha$ -2b variant was produced in mammalian cells.

Figure 6(C) presents the characterization of the E113H IFN $\alpha$ -2b variant treated with blood lysate. The percent of residual (anti-viral) activity for the variant (black line and white circles) after treatment with  
25 blood lysate was compared to the treated wild-type IFN $\alpha$ -2b (dashed line and black squares). For this experiment, the E113H IFN $\alpha$ -2b variant was produced in mammalian cells.

Figure 6(D) presents the characterization of the E113H IFN $\alpha$ -2b variant treated with serum. The percent of residual (anti-viral) activity for

-9-

the variant (black line and white circles) after treatment with serum was compared to the treated wild-type IFN $\alpha$ -2b (dashed line and black squares). For this experiment, the E113H IFN $\alpha$ -2b variant was produced in mammalian cells.

5            Figures 6(E) and 6(F) provide graphs indicating the levels of protection against in vitro proteolysis for IFN $\alpha$ -2b variants produced in bacteria. In Figures 6(E) and 6(F), the vertical axis indicates the relative level of non-proteolized protein and the horizontal axis indicates time in hours. The percent of residual (anti-viral) activity for the variants (gray  
10 circles with continuous lines) after treatment were compared to the treated wild-type IFN $\alpha$ -2b (solid circles with dashed lines).

Figure 6(G) provides graphs indicating the in vitro potency for antiviral activity, for IFN $\alpha$ -2b variants produced in bacteria. The vertical axis indicates the level of antiviral activity and the horizontal axis  
15 indicates concentration of the variants at which each level of activity is achieved. The activity for the variants (continuous line with gray circles) was compared to that of the wild-type IFN $\alpha$ -2b (black triangles with dashed lines). The potency for each variant was calculated from the graphs as the concentration at the inflection point of the respective  
20 curves. Figure 6(T) shows the value of potency obtained for each variant tested compared to the wild type IFN $\alpha$ .

Figure 6(H) provides the in vitro potency for anti-proliferation activity, for IFN $\alpha$ -2b variants produced in bacteria. The activity for the variants was compared to that of the wild-type IFN $\alpha$ -2b in serial dilution  
25 experiments where the anti-proliferation activity was measured for a number of dilutions for each variant. Potency was calculated from the graphs as the concentration at the inflection point of the respective curves. The figure shows the value of potency obtained for each variant tested and in comparison to the wild type IFN $\alpha$ .

-10-

Figures 6(I) to 6(N) provide graphs indicating the pharmacokinetics in mice following subcutaneous injection of IFN $\alpha$ -2b variants produced in bacteria. The vertical axis indicates the level of antiviral activity in blood and the horizontal axis indicates the time after injection at which the level of antiviral activity is determined. The pharmacokinetics of the variants (in gray solid circles with gray continuous lines) was compared to that of the wild-type IFN $\alpha$ -2b (in black with dashed lines) and of a pegylated derivative (Pegasys, Roche) (36 $\mu$ g/ml open triangles with continuous black lines; and 18  $\mu$ g/ml open circles with continuous black lines); and vehicle (gray solid triangles with continuous gray lines). The Area Under the Curve (AUC) for each variant was calculated from the graphs and is shown in 6(U).

Figure 6(O) provides graphs indicating the levels of protection against *in vitro* proteolysis for IFN $\beta$  variants produced in mammalian cells. Figure 6(N), the vertical axis indicates the relative level of non-proteolized protein and the horizontal axis indicates time in hours. The percent of residual (anti-viral) activity for the variants after treatment were compared to the treated wild-type IFN $\beta$ .

Figures 6(P) to 6(S) provide graphs indicating the *in vitro* potency for either antiviral activity (6(P) and 6(Q)) or anti-proliferative activity (6(R) and 6(S)), for a number of IFN $\beta$  variants produced in mammalian cells. The vertical axis indicates the level of (antiviral or anti-proliferation) activity and the horizontal axis indicates the concentration of the variants at which each level of activity is achieved. The activity for the variants (6(Q) and 6(S)) was compared to that of the wild-type IFN $\beta$  (6(P) and 6(R)). The activity obtained with either no previous treatment or by treating the variants with proteases prior to the activity test is shown.

Figure 6(T) provides a comparison of antiviral activity (potency), anti-proliferation activity (potency), number of mutations present and AUC

-11-

(from PK) for a number of IFN $\alpha$ -2b and in comparison with the wild-type IFN $\alpha$ -2b.

Figure 6(U) provides IFN units injected and protein injected ( $\mu$ g/ml) for the data in Figure 6(T).

- 5        Figure 7(A) depicts a top view ribbon representation of IFN $\alpha$ -2b structure obtained from the NMR structure of IFN $\alpha$ -2 $\alpha$  (PDB code 1ITF). Residues represented in "space filling" define (1) the "receptor binding region" based on either our "alanine scanning" analysis or on studies by Piehler *et al.*, *J. Biol. Chem.*, 275:40425-40433, 2000, and Roisman *et al.*, *Proc. Natl. Acad. Sci. USA*, 98:13231-13236, 2001 (in light-gray and  
10        *al.*, *Proc. Natl. Acad. Sci. USA*, 98:13231-13236, 2001 (in light-gray and dark-gray, respectively), and (2) replacing residues (LEADs) for resistance to proteolysis (in black).

Figure 7(B) depicts a side view ribbon representation of IFN $\alpha$ -2b structure (PDB code 1ITF). Residue representation is as in FIG7A.

- 15        Figure 8(A) schematizes the identification of homologous amino acid positions between a number of cytokines and the LEAD mutants of IFN $\alpha$ -2b using 3-dimensional scanning (also referred to herein as based on "structure-based homology" methods or "structural homology" methods).

- 20        Figure 8(B) illustrates a structural overlapping between human interferon  $\alpha$ -2b obtained from the NMR structure of IFN $\alpha$ -2a (PDB code 1ITF) and human interferon  $\beta$  (PDB code 1AU1) using Swiss Pdb Viewer.

Figure 8(C) illustrates a structural overlapping between human interferon  $\alpha$ -2b obtained from the NMR structure of IFN $\alpha$ -2a (PDB code 1ITF) and erythropoietin (PDB code 1BUY) using Swiss Pdb Viewer.

- 25        Figure 8(D) illustrates a structural overlapping between human interferon  $\alpha$ -2b obtained from the NMR structure of IFN $\alpha$ -2a (PDB code 1ITF) and granulocyte-colony stimulating factor (PDB code 1CD9) using Swiss Pdb Viewer.



-12-

Figure 9 illustrates a structural alignment of a number of cytokines and interferon  $\alpha$ -2b sequences. Bold underlined residues define the region on each cytokine sequence that based on structural homology comparison corresponds to the structurally-related mutations found on the LEADs for  
5 protease resistance of IFN $\alpha$ -2b.

Figure 10(A) shows the antiviral activity of interferon  $\alpha$ -2b mutants generated by alanine-scanning analysis used for protein redesign. Plotted symbols for wild type and variants of interferon  $\alpha$ -2b are indicated in the inset.

10 Figure 10(B) displays cell proliferation after treatment with interferon  $\alpha$ -2b mutants obtained by alanine-scanning analysis. Plotted symbols for wild type and variants of interferon  $\alpha$ -2b are indicated in the inset.

Figure 10(C) displays the correlation between the antiviral activity  
15 and cell proliferation activity of interferon  $\alpha$ -2b mutants obtained by alanine-scanning analysis.

Figure 11 Candidate glycosylation sites for interferon  $\alpha$ -2b stabilization and redesign thereof.

Figure 12 (A) shows the is-HIT residue positions and type of  
20 replacing amino acids selected to generate modified protein sequences of interferon  $\beta$  (corresponding to SEQ ID Nos: 233-289), based on 3D-scanning (structural homology method), including PAM250 analysis.

Figure 12 (B) displays the is-HIT residue positions and type of  
replacing amino acids selected to generate modified protein sequences of  
25 interferon gamma (corresponding to SEQ ID Nos: 290-311), based on structural homology and PAM250 analysis.

Figure 12 (C) shows the is-HIT residue positions and type of  
replacing amino acids selected to generate modified protein sequences of

-13-

interleukin-10 (corresponding to SEQ ID Nos: 312-361), based on structural homology and PAM250 analysis.

Figure 12 (D) displays the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of ciliary neurotrophic factor (corresponding to SEQ ID Nos: 684-728), based on structural homology and PAM250 analysis.

Figure 12 (E) shows the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of granulocyte-colony stimulating factor (corresponding to SEQ ID Nos: 631-662), based on structural homology and PAM250 analysis.

Figure 12 (F) displays the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of human growth hormone (corresponding to SEQ ID Nos: 850-895), based on structural homology and PAM250 analysis.

Figure 12 (G) shows the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of interleukin-12 (corresponding to SEQ ID Nos: 794-849), based on structural homology and PAM250 analysis.

Figure 12 (H) displays the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of interleukin-6 (corresponding to SEQ ID Nos: 896-939), based on structural homology and PAM250 analysis.

Figure 12 (I) shows the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of leptin (corresponding to SEQ ID Nos: 663-683), based on structural homology and PAM250 analysis.

Figure 12 (J) displays the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of

-14-

leukemia inhibitory factor (corresponding to SEQ ID Nos: 729-760), based on structural homology and PAM250 analysis.

Figure 12 (K) shows the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of  
5 oncostatin M (corresponding to SEQ ID Nos: 761-793), based on structural homology and PAM250 analysis.

Figure 12 (L) displays the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of erythropoietin (corresponding to SEQ ID Nos: 940-977), based on  
10 structural homology and PAM250 analysis.

Figure 12 (M) shows the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of Flt3 ligand (corresponding to SEQ ID Nos: 401-428), based on structural homology and PAM250 analysis.

15 Figure 12 (N) displays the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of granulocyte-macrophage colony-stimulating factor (corresponding to SEQ ID Nos: 362-400), based on structural homology and PAM250 analysis.

Figure 12 (O) shows the is-HIT residue positions and type of  
20 replacing amino acids selected to generate modified protein sequences of interleukin-13 (corresponding to SEQ ID Nos: 603-630), based on structural homology and PAM250 analysis.

Figure 12 (P) displays the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of  
25 interleukin-2 (corresponding to SEQ ID Nos: 429-476), based on structural homology and PAM250 analysis.

Figure 12 (Q) shows the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of

-15-

interleukin-3 (corresponding to SEQ ID Nos: 477-498), based on structural homology and PAM250 analysis.

Figure 12 (R) displays the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of interleukin-4 (corresponding to SEQ ID Nos: 543-567), based on structural homology and PAM250 analysis.

Figure 12 (S) shows the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of interleukin-5 (corresponding to SEQ ID Nos: 568-602), based on structural homology and PAM250 analysis.

Figure 12 (T) displays the is-HIT residue positions and type of replacing amino acids selected to generate modified protein sequences of stem cell factor (corresponding to SEQ ID Nos: 499-542), based on structural homology and PAM250 analysis.

## 15 DETAILED DESCRIPTION

### OUTLINE

- A. Definitions
- B. Directed Evolution
  - 1) Pure Random Mutagenesis
  - 2) Restricted Random Mutagenesis
  - 3) Non-restricted Rational mutagenesis
- C. 2-Dimensional Rational Scanning (2D scanning)
  - 1) Identifying In-silico HITS
  - 2) Identifying Replacing Amino Acids
    - (a) Percent Accepted Mutation (PAM)
      - (i) PAM Analysis
      - (ii) PAM250
    - (b) Jones and Gonnet
    - (c) Fitch and Feng
    - (d) McLachlan, Grantham and Miyata
    - (e) Rao
    - (f) Risler
    - (g) Johnson
    - (h) Block Substitution Matrix (BLOSUM)
  - 3) Physical Construction of Mutant Proteins and Biological Assays
- D. 2-Dimensional Scanning of Proteins for Increased Resistance to Proteolysis
- E. Rational Evolution of IFN $\alpha$ -2b For Increased Resistance to Proteolysis

-16-

- 1) Modified IFN $\alpha$ -2b and IFN $\alpha$ -2a Proteins with Single Amino Acid Substitutions (is-HITs)
- 2) LEAD identification
- 3) N-glycosylation Site Addition
- 5 F. Protein Redesign
- G. 3D-scanning and Its Use for Modifying Cytokines
  - 1) Homology
  - 2) 3D-scanning (Structural Homology)
  - 3) Application of the 3D-scanning method to Cytokines
    - (a) Structurally Homologous Interferon Mutants
    - (b) Structurally Homologous Cytokine Mutants
- 10 H. Rational Evolution of IFN $\beta$  For Increased Resistance to Proteolysis Modified IFN $\beta$  Proteins with Single Amino Acid Substitutions
- I. Super-LEADs and Additive Directional Mutagenesis (ADM).
  - 15 1) Additive Directional Mutagenesis
  - 2) Multi-overlapped Primer Extensions
- J. Uses of the Mutant IFN $\alpha$ , IFN $\beta$ -2b Genes and Cytokines in Therapeutic Methods
  - 20 1) Fusion Proteins
  - 2) Nucleic Acid Molecules for Expression
  - 3) Formulation of Optimized Cytokines
- K. Examples

#### A. Definitions

Unless defined otherwise, all technical and scientific terms used  
 25 herein have the same meaning as is commonly understood by one of skill  
 in the art to which the invention(s) belong. All patents, patent  
 applications, published applications and publications, Genbank sequences,  
 websites and other published materials referred to throughout the entire  
 disclosure herein, unless noted otherwise, are incorporated by reference  
 30 in their entirety. In the event that there is a plurality of definitions for  
 terms herein, those in this section prevail. Where reference is made to a  
 URL or other such identifier or address, it understood that such identifiers  
 can change and particular information on the internet can come and go,  
 but equivalent information can be found by searching the internet.  
 35 Reference thereto evidences the availability and public dissemination of  
 such information.

As used herein, biological activity of a protein refers to any activity  
 manifested by the protein *in vivo*.

-17-

As used herein, "a directed evolution method" refers to methods that "adapt" either natural proteins, synthetic proteins or protein domains to work in new or existing natural or artificial chemical or biological environments and/or to elicit new functions and/or to increase or decrease a given activity, and/or to modulate a given feature. Exemplary directed evolution methods include pure random mutagenesis methods; restricted random mutagenesis methods; and non-restricted rational mutagenesis methods, such as the rational directed evolution method described in co-pending U.S. application Serial No. 10/022,249; and the 2-dimensional rational scanning method provided herein.

As used herein, two dimensional rational mutagenesis scanning (2D scanning) refers to the processes provided herein in which two dimensions of a particular protein sequence are scanned: (1) one dimension is to identify specific amino acid residues along the protein sequence to replace with different amino acids, referred to as is-HIT target positions, and (2) the second dimension is the amino acid type selected for replacing the particular is-HIT target, referred to as the replacing amino acid.

As used herein, *in silico* refers to research and experiments performed using a computer. In silico methods include, but are not limited to, molecular modeling studies, and biomolecular docking experiments.

As used herein, "is-HIT" refers to an *in silico* identified amino acid position along a target protein sequence that has been identified based on i) the particular protein properties to be evolved, ii) the protein's amino acid sequence, and/or iii) the known properties of the individual amino acids. These is-HIT loci on the protein sequence are identified without use of experimental biological methods. For example, once the protein feature(s) to be optimized is (are) selected, diverse sources of information

-18-

or previous knowledge (i.e., protein primary, secondary or tertiary structures, literature, patents) are exploited to determine those amino acid positions that may be amenable to improved protein fitness by replacement with a different amino acid. This step utilizes protein  
5 analysis "*in silico*." All possible candidate amino acid positions along a target proteins primary sequence that might be involved in the feature being evolved are referred to herein as "*in silico* HITs" ("is-HITs"). The collection (library), of all is-HITs identified during this step represents the first dimension (target residue position) of the two-dimensional scanning  
10 methods provided herein.

As used herein, "amenable to providing the evolved predetermined property or activity," in the context of identifying is-HITs, refers to an amino acid position on a protein that is contemplated, based on *in silico* analysis, to possess properties or features that when replaced would  
15 result in the desired activity being evolved. The phrase "amenable to providing the evolved predetermined property or activity," in the context of identifying replacement amino acids, refers to a particular amino acid type that is contemplated, based on *in silico* analysis, to possess properties or features that when used to replace the original amino acid in  
20 the unmodified starting protein would result in the desired activity being evolved.

As used herein, high-throughput screening (HTS) refers to processes that test a large number of samples, such as samples of test proteins or cells containing nucleic acids encoding the proteins of interest  
25 to identify structures of interest or the identify test compounds that interact with the variant proteins or cells containing them. HTS operations are amenable to automation and are typically computerized to handle sample preparation, assay procedures and the subsequent processing of large volumes of data.

-19-

As used herein, the term "restricted," when used in the context of the identification of is-HIT amino acid positions along the protein sequence selected for amino acid replacement and/or the identification of replacing amino acids, means that fewer than all amino acids on the protein-backbone are selected for amino acid replacement; and/or fewer than all of the remaining 19 amino acids available to replace the original amino acid present in the unmodified starting protein are selected for replacement. In particular embodiments of the methods provided herein, the is-HIT amino acid positions are restricted, such that fewer than all amino acids on the protein-backbone are selected for amino acid replacement. In other embodiments, the replacing amino acids are restricted, such that fewer than all of the remaining 19 amino acids available to replace the native amino acid present in the unmodified starting protein are selected as replacing amino acids. In a particular embodiment, both of the scans to identify is-HIT amino acid positions and the replacing amino acids are restricted, such that fewer than all amino acids on the protein-backbone are selected for amino acid replacement and fewer than all of the remaining 19 amino acids available to replace the native amino acid are selected for replacement.

As used herein, "candidate LEADs," are mutant proteins that are contemplated as potentially having an alteration in any attribute, chemical, physical or biological property in which such alteration is sought. In the methods herein, candidate LEADs are generally generated by systematically replacing is-HITS loci in a protein or a domain thereof with typically a restricted subset, or all, of the remaining 19 amino acids, such as obtained using PAM analysis. Candidate LEADs can be generated by other methods known to those of skill in the art tested by the high throughput methods herein.



-20-

As used herein, "LEADs" are "candidate LEADs" whose activity has been demonstrated to be optimized or improved for the particular attribute, chemical, physical or biological property. For purposes herein a "LEAD" typically has activity with respect to the function of interest that  
5 differs by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200% or more from the unmodified and/or wild type (native) protein. In certain embodiments, the change in activity is at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%, of the activity of the unmodified target protein. In other embodiments, the  
10 change in activity is not more than about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%, of the activity of the unmodified target protein. In yet other embodiments, the change in activity is at least about 2 times, 3 times, 4 times, 5 times, 6 times, 7 times, 8 times, 9 times, 10 times, 20 times, 30 times, 40 times, 50 times, 60 times, 70 times, 80  
15 times, 90 times, 100 times, 200 times, 300 times, 400 times, 500 times, 600 times, 700 times, 800 times, 900 times, 1000 times, or more greater than the activity of the unmodified target protein. The desired alteration, which can be either an increase or a reduction in activity, will depend upon the function or property of interest (e.g.,  $\pm 10\%$ ,  $\pm 20\%$ , etc.). The  
20 LEADs may be further optimized by replacement of a plurality (2 or more) of "is-HIT" target positions on the same protein molecule to generate "super-LEADs."

As used herein, the term "super-LEAD" refers to protein mutants (variants) obtained by combining the single mutations present in two or  
25 more of the LEAD molecules into a single protein molecule. Accordingly, in the context of the modified proteins provided herein, the phrase "proteins comprising one or more single amino acid replacements" encompasses any combination of two or more of the mutations described herein for a respective protein. For example, the modified proteins

-21-

provided herein having one or more single amino acid replacements can have can have any combination of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more of the amino acid replacements at the disclosed replacement positions. The collection of super-LEAD mutant  
5 molecules is generated, tested and phenotypically characterized one-by-one in addressable arrays. Super-LEAD mutant molecules are such that each molecule contains a variable number and type of LEAD mutations. Those molecules displaying further improved fitness for the particular feature being evolved, are referred to as super-LEADs. Super-LEADs can  
10 be generated by other methods known to those of skill in the art and tested by the high throughput methods herein. For purposes herein a super-LEAD typically has activity with respect to the function of interest that differs from the improved activity of a LEAD by a desired amount, such as at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%,  
15 100%, 150%, 200% or more from at least one of the LEAD mutants from which it is derived. As with LEADs, the change in the activity for super-LEADs is dependent upon the activity that is being "evolved." The desired alteration, which can be either an increase or a reduction in activity, will depend upon the function or property of interest.

20 As used herein, a recitation that modified protein has more antiviral activity (or other activity) than antiproliferative activity (or another activity) compared to the unmodified cytokine, is comparing the absolute value of the change in each activity compared to wild type.

As used herein, the phrase "altered loci" refers to the is-HIT amino  
25 acid positions in the LEADs or super-LEADs that are replaced with different replacing amino acids, resulting in the desired altered phenotype or activity.

As used herein, an exposed residue presents more than 15% of its surface exposed to the solvent.

-22-

As used herein, the phrase "structural homology" refers to the degree of coincidence in space between two or more protein backbones. Protein backbones that adopt the same protein structure, fold and show similarity upon three-dimensional structural superposition in space can be

5 considered structurally homologous. Structural homology is not based on sequence homology, but rather on three-dimension homology. Two amino acids in two different proteins said to be homologous based on structural homology between those proteins, do not necessarily need to be in sequence-based homologous regions. For example, protein

10 backbones that have a root mean squared (RMS) deviation of less than 3.5, 3.0, 2.5, 2.0, 1.7 or 1.5 angstrom at a given space position or defined region between each other can be considered to be structurally homologous in that region, and are referred to herein as having a "high coincidence" between their backbones. It is contemplated herein that

15 substantially equivalent (e.g., "structurally related") amino acid positions that are located on two or more different protein sequences that share a certain degree of structural homology will have comparable functional tasks; also referred to herein as "structurally homologous loci." These two amino acids can then be said to be "structurally similar" or

20 "structurally related" with each other, even if their precise primary linear positions on the amino acid sequences, when these sequences are aligned, do not match with each other. Amino acids that are "structurally related" can be far away from each other in the primary protein sequences, when these sequences are aligned following the rules of

25 classical sequence homology.

As used herein, a structural homolog is a protein that is generated by structural homology.

As used herein, the phrase "unmodified target protein," "unmodified protein" or "unmodified cytokine," or grammatical variations

-23-

thereof, refers to a starting protein that is selected for optimization using the methods provided herein. The starting unmodified target protein can be the naturally occurring, wild type form of a protein. In addition, the starting unmodified target protein may have previously been altered or

5 mutated, such that it differs from the native wild type isoform, but is nonetheless referred to herein as an starting unmodified target protein relative to the subsequently modified proteins produced herein. Thus, existing proteins known in the art that have previously been modified to have a desired increase or decrease in a particular biological activity

10 compared to an unmodified reference protein can be selected and used herein as the starting "unmodified target protein." For example, a protein that has been modified from its native form by one or more single amino acid changes and possesses either an increase or decrease in a desired activity, such as resistance to proteolysis, can be utilized with the

15 methods provided herein as the starting unmodified target protein for further optimization of either the same or a different biological activity.

Likewise, existing proteins known in the art that have previously been modified to have a desired increase or decrease in a particular biological activity compared to an unmodified reference protein can be

20 selected and used herein for identification of structurally homologous loci on other structurally homologous target proteins. For example, a protein that has been modified by one or more single amino acid changes and possesses either an increase or decrease in a desired activity, such as resistance to proteolysis, can be utilized with the methods provided herein

25 to identify on structurally homologous target proteins, corresponding structurally homologous loci that can be replaced with suitable replacing amino acids and tested for either an increase or decrease in the desired biological activity.

-24-

As used herein, the phrase "only one amino acid replacement occurs on each target protein" refers to the modification of a target protein, such that it differs from the unmodified form of the target protein by a single amino acid change. For example, in one embodiment, 5 mutagenesis is performed by the replacement of a single amino acid residue at only one is-HIT target position on the protein backbone (e.g., "one-by-one" in addressable arrays), such that each individual mutant generated is the single product of each single mutagenesis reaction. The single amino acid replacement mutagenesis reactions are repeated for 10 each of the replacing amino acids selected at each of the is-HIT target positions. Thus, a plurality of mutant protein molecules are produced, whereby each mutant protein contains a single amino acid replacement at only one of the is-HIT target positions.

As used herein, the phrase "pseudo-wild type," in the context of 15 single or multiple amino acid replacements, are those amino acids that, while different from the original, such as native, amino acid at a given amino acid position, can replace the native one at that position without introducing any measurable change in a particular protein activity. A population of sets of nucleic acid molecules encoding a collection of 20 mutant molecules is generated and phenotypically characterized such that proteins with amino acid sequences different from the original amino acid, but that still elicit substantially the same level (i.e., at least 10%, 50%, 70%, 90%, 95%, 100%, depending upon the protein) and type of desired activity as the original protein are selected.

25 As used herein, biological and pharmacological activity includes any activity of a biological pharmaceutical agent and includes, but is not limited to, resistance to proteolysis, biological efficiency, transduction efficiency, gene/transgene expression, differential gene expression and induction activity, titer, progeny productivity, toxicity, cytotoxicity,

-25-

immunogenicity, cell proliferation and/or differentiation activity, anti-viral activity, morphogenetic activity, teratogenetic activity, pathogenetic activity, therapeutic activity, tumor suppressor activity, ontogenetic activity, oncogenetic activity, enzymatic activity, pharmacological  
5 activity, cell/tissue tropism and delivery.

As used herein, "output signal" refers to parameters that can be followed over time and, if desired, quantified. For example, when a recombinant protein is introduced into a cell, the cell containing the recombinant protein undergoes a number of changes. Any such change  
10 that can be monitored and used to assess the transformation or transfection, is an output signal, and the cell is referred to as a reporter cell; the encoding nucleic acid is referred to as a reporter gene, and the construct that includes the encoding nucleic acid is a reporter construct. Output signals include, but are not limited to, enzyme activity,  
15 fluorescence, luminescence, amount of product produced and other such signals. Output signals include expression of a gene or gene product, including heterologous genes (transgenes) inserted into the plasmid virus. Output signals are a function of time ("t") and are related to the amount of protein used in the composition. For higher concentrations of protein,  
20 the output signal can be higher or lower. For any particular concentration, the output signal increases as a function of time until a plateau is reached. Output signals can also measure the interaction between cells, expressing heterologous genes, and biological agents

As used herein, the activity of an IFN $\alpha$ -2b or IFN $\alpha$ -2a protein refers  
25 to any biological activity that can be assessed. In particular, herein, the activity assessed for the IFN $\alpha$ -2b or IFN $\alpha$ -2a proteins is resistance to proteolysis, antiviral activity and cell proliferation activity.

-26-

As used herein, the Hill equation is a mathematical model that relates the concentration of a drug (*i.e.*, test compound or substance) to the response measured

$$y = \frac{y_{\max} [D]^n}{[D]^n + [D_{50}]^n},$$

where  $y$  is the variable measured, such as a response, signal,  $y_{\max}$  is the maximal response achievable,  $[D]$  is the molar concentration of a drug,  $[D_{50}]$  is the concentration that produces a 50% maximal response to the drug,  $n$  is the slope parameter, which is 1 if the drug binds to a single site and with no cooperativity between or among sites. A Hill plot is  $\log_{10}$  of the ratio of ligand-occupied receptor to free receptor vs.  $\log [D]$  (M). The slope is  $n$ , where a slope of greater than 1 indicates cooperativity among binding sites, and a slope of less than 1 can indicate heterogeneity of binding. This general equation has been employed for assessing interactions in complex biological systems (see, published International PCT application No. WO 01/44809 based on PCT No. PCT/FR00/03503, see also, the EXAMPLES).

As used herein, in the Hill-based analysis (published International PCT application No. WO 01/44809 based on PCT No. PCT/FR00/03503), the parameters,  $\pi, \kappa, \tau, \epsilon, \eta, \theta$ , are as follows:

- $\pi$  is the potency of the biological agent acting on the assay (cell-based) system;
- $\kappa$  is the constant of resistance of the assay system to elicit a response to a biological agent;
- $\epsilon$  is the global efficiency of the process or reaction triggered by the biological agent on the assay system;
- $\tau$  is the apparent titer of the biological agent;
- $\theta$  is the absolute titer of the biological agent; and

-27-

$\eta$  is the heterogeneity of the biological process or reaction.

In particular, as used herein, the parameters  $\pi$  (potency) or  $\kappa$  (constant of resistance) are used to respectively assess the potency of a test agent to produce a response in an assay system and the resistance  
5 of the assay system to respond to the agent.

As used herein,  $\epsilon$  (efficiency), is the slope at the inflexion point of the Hill curve (or, in general, of any other sigmoidal or linear approximation), to assess the efficiency of the global reaction (the biological agent and the assay system taken together) to elicit the  
10 biological or pharmacological response.

As used herein,  $\tau$  (apparent titer) is used to measure the limiting dilution or the apparent titer of the biological agent.

As used herein,  $\theta$  (absolute titer), is used to measure the absolute limiting dilution or titer of the biological agent.

15 As used herein,  $\eta$  (heterogeneity) measures the existence of discontinuous phases along the global reaction, which is reflected by an abrupt change in the value of the Hill coefficient or in the constant of resistance.

As used herein, a population of sets of nucleic acid molecules  
20 encoding a collection (library) of mutants refers to a collection of plasmids or other vehicles that carry (encode) the gene variants, such that individual plasmids or other individual vehicles carry individual gene variants. Each element (member) of the collection is physically separated from the others, such as individually in an appropriate addressable array,  
25 and has been generated as the single product of an independent mutagenesis reaction. When a collection (library) of such proteins is contemplated, it will be so-stated.

As used herein, a "reporter cell" is the cell that "reports," *i.e.*, undergoes the change, in response to a condition, such as, for example,



-28-

exposure to a protein or a virus or to a change in its external or internal environment.

As used herein, "reporter" or "reporter moiety" refers to any moiety that allows for the detection of a molecule of interest, such as a protein expressed by a cell. Reporter moieties include, but are not limited to, for example, fluorescent proteins, such as red, blue and green fluorescent proteins; LacZ and other detectable proteins and gene products. For expression in cells, nucleic acid encoding the reporter moiety can be expressed as a fusion protein with a protein of interest or under the control of a promoter of interest.

As used herein, phenotype refers to the physical, physiological or other manifestation of a genotype (a sequence of a gene). In methods herein, phenotypes that result from alteration of a genotype are assessed.

As used herein, "activity" means in the largest sense of the term any change in a system (either biological, chemical or physical system) of any nature (changes in the amount of product in an enzymatic reaction, changes in cell proliferation, in immunogenicity, in toxicity) caused by a protein or protein mutant when they interact with that system. In addition, the term "activity," "higher activity" or "lower activity" as used herein in reference to resistance to proteases, proteolysis, incubation with serum or with blood, means the ratio or residual biological (antiviral) activity between "after" protease/blood or serum treatment and "before" protease/blood or serum treatment.

As used herein, activity refers to the function or property to be evolved. An active site refers to a site(s) responsible or that participates in conferring the activity or function. The activity or active site evolved (the function or property and the site conferring or participating in conferring the activity) can have nothing to do with natural activities of a

-29-

protein. For example, it could be an 'active site' for conferring immunogenicity (immunogenic sites or epitopes) on a protein.

As used herein, the amino acids, which occur in the various amino acid sequences appearing herein, are identified according to their known, three-letter or one-letter abbreviations (see, Table 1). The nucleotides, which occur in the various nucleic acid fragments, are designated with the standard single-letter designations used routinely in the art.

As used herein, amino acid residue refers to an amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are presumed to be in the "L" isomeric form. Residues in the "D" isomeric form, which are so-designated, can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH<sub>2</sub> refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxyl terminus of a polypeptide. In keeping with standard polypeptide nomenclature described in *J. Biol. Chem.*, 243:3552-3559, 1969, and adopted at 37 C.F.R. §§ 1.821 - 1.822, abbreviations for amino acid residues are shown in Table 1:

**Table 1**  
Table of Correspondence

SYMBOL		
1-Letter	3-Letter	AMINO ACID
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine

-30-

5	SYMBOL		
	S	Ser	serine
	I	Ile	isoleucine
	L	Leu	leucine
	T	Thr	threonine
	V	Val	valine
	P	Pro	proline
	K	Lys	lysine
	H	His	histidine
10	Q	Gln	glutamine
	E	Glu	glutamic acid
	Z	Glx	Glu and/or Gln
	W	Trp	tryptophan
15	R	Arg	arginine
	D	Asp	aspartic acid
	N	Asn	asparagine
	B	Asx	Asn and/or Asp
	C	Cys	cysteine
	X	Xaa	Unknown or other

20 It should be noted that all amino acid residue sequences represented herein by formulae have a left to right orientation in the conventional direction of amino-terminus to carboxyl-terminus. In addition, the phrase "amino acid residue" is broadly defined to include the amino acids listed in the Table of Correspondence (Table 1) and modified

25 and unusual amino acids, such as those referred to in 37 C.F.R. §§ 1.821-1.822, and incorporated herein by reference. Furthermore, it should be noted that a dash at the beginning or end of an amino acid

-31-

residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues or to an amino-terminal group such as NH<sub>2</sub> or to a carboxyl-terminal group such as COOH.

As used herein, nucleic acids include DNA, RNA and analogs thereof, including protein nucleic acids (PNA) and mixture thereof. Nucleic acids can be single or double stranded. When referring to probes or primers, optionally labeled, with a detectable label, such as a fluorescent or radiolabel, single-stranded molecules are contemplated. Such molecules are typically of a length such that they are statistically  
10 unique of low copy number (typically less than 5, generally less than 3) for probing or priming a library. Generally a probe or primer contains at least 14, 16 or 30 contiguous of sequence complementary to or identical a gene of interest. Probes and primers can be 10, 14, 16, 20, 30, 50, 100 or more nucleic acid bases long.

15 Therefore, as used herein, the term "identity" represents a comparison between a test and a reference polypeptide or polynucleotide. For example, a test polypeptide can be defined as any polypeptide that is 90% or more identical to a reference polypeptide.

As used herein, "corresponding structurally-related" positions on  
20 two or more proteins, such as the IFN $\alpha$ -2b protein and other cytokines, refers those amino acid positions determined based upon structural homology to maximize tri-dimensional overlapping between proteins.

As used herein, the term at least "90% identical to" refers to percent identities from 90 to 100% relative to the reference polypeptides.  
25 Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polypeptide length of 100 amino acids are compared. No more than 10% (i.e., 10 out of 100) amino acids in the test polypeptide differ from that of the reference polypeptides. Similar comparisons can be made between a test and

-32-

reference polynucleotides. Such differences can be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they can be clustered in one or more locations of varying length up to the maximum allowable, e.g., 10/100 amino acid difference  
5 (approximately 90% identity). Differences are defined as nucleic acid or amino acid substitutions, or deletions.

As used herein, the phrase "sequence-related proteins" refers to proteins that have at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% amino acid identity or homology with  
10 each other.

As used herein, families of non-related proteins or "sequence-non-related proteins" refers to proteins that have less than 50%, less than 40%, less than 30%, less than 20% amino acid identity or homology with each other.

15 As used herein, it also is understood that the terms "substantially identical" or "similar" varies with the context as understood by those skilled in the relevant art.

As used herein, heterologous or foreign nucleic acid, such as DNA and RNA, are used interchangeably and refer to DNA or RNA that does  
20 not occur naturally as part of the genome in which it is present or which is found in a location or locations in the genome that differ from that in which it occurs in nature. Heterologous nucleic acid is generally not endogenous to the cell into which it is introduced, but has been obtained from another cell or prepared synthetically. Generally, although not  
25 necessarily, such nucleic acid encodes RNA and proteins that are not normally produced by the cell in which it is expressed. Heterologous DNA herein encompasses any DNA or RNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which it is expressed. Heterologous DNA and RNA can also encode RNA

-33-

or proteins that mediate or alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes. Examples of heterologous nucleic acid include, but are not limited to, nucleic acid that encodes traceable marker proteins, such as a protein that confers drug resistance, nucleic acid that encodes therapeutically effective substances, such as anti-cancer agents, enzymes and hormones, and DNA that encodes other types of proteins, such as antibodies.

Hence, herein heterologous DNA or foreign DNA, includes a DNA molecule not present in the exact orientation and position as the counterpart DNA molecule found in the genome. It can also refer to a DNA molecule from another organism or species (*i.e.*, exogenous).

As used herein, a therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of disease.

As used herein, isolated with reference to a nucleic acid molecule or polypeptide or other biomolecule means that the nucleic acid or polypeptide has separated from the genetic environment from which the polypeptide or nucleic acid were obtained. It can also mean altered from the natural state. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated," as the term is employed herein. Thus, a polypeptide or polynucleotide produced and/or contained within a recombinant host cell is considered isolated. Also intended as an "isolated polypeptide" or an "isolated polynucleotide" are polypeptides or polynucleotides that have been purified, partially or substantially, from a recombinant host cell or from a native source. For example, a recombinantly produced version of a compound can be substantially purified by the one-step method

-34-

described in Smith *et al.*, *Gene*, 67:31-40, 1988. The terms isolated and purified are sometimes used interchangeably.

Thus, by "isolated" is meant that the nucleic is free of the coding sequences of those genes that, in the naturally-occurring genome of the organism (if any) immediately flank the gene encoding the nucleic acid of interest. Isolated DNA can be single-stranded or double-stranded, and can be genomic DNA, cDNA, recombinant hybrid DNA, or synthetic DNA. It can be identical to a starting DNA sequence, or can differ from such sequence by the deletion, addition, or substitution of one or more nucleotides.

Isolated or purified as it refers to preparations made from biological cells or hosts means any cell extract containing the indicated DNA or protein including a crude extract of the DNA or protein of interest. For example, in the case of a protein, a purified preparation can be obtained following an individual technique or a series of preparative or biochemical techniques and the DNA or protein of interest can be present at various degrees of purity in these preparations. The procedures can include for example, but are not limited to, ammonium sulfate fractionation, gel filtration, ion exchange change chromatography, affinity chromatography, density gradient centrifugation and electrophoresis.

A preparation of DNA or protein that is "substantially pure" or "isolated" should be understood to mean a preparation free from naturally occurring materials with which such DNA or protein is normally associated in nature. "Essentially pure" should be understood to mean a "highly" purified preparation that contains at least 95% of the DNA or protein of interest.

A cell extract that contains the DNA or protein of interest should be understood to mean a homogenate preparation or cell-free preparation obtained from cells that express the protein or contain the DNA of

-35-

interest. The term "cell extract" is intended to include culture media, especially spent culture media from which the cells have been removed.

As used herein, "a targeting agent" refers to any molecule that can bind another target-molecule, such as an antibody, receptor, or ligand.

5 As used herein, receptor refers to a biologically active molecule that specifically binds to (or with) other molecules. The term "receptor protein" can be used to more specifically indicate the proteinaceous nature of a specific receptor.

10 As used herein, recombinant refers to any progeny formed as the result of genetic engineering.

As used herein, a promoter region refers to the portion of DNA of a gene that controls transcription of the DNA to which it is operatively linked. The promoter region includes specific sequences of DNA that are sufficient for RNA polymerase recognition, binding and transcription  
15 initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of the RNA polymerase. These sequences can be *cis* acting or can be responsive to *trans* acting factors. Promoters, depending upon the nature  
20 of the regulation, can be constitutive or regulated.

As used herein, the phrase "operatively linked" generally means the sequences or segments have been covalently joined into one piece of DNA, whether in single or double stranded form, whereby control or regulatory sequences on one segment control or permit expression or  
25 replication or other such control of other segments. The two segments are not necessarily contiguous. For gene expression a DNA sequence and a regulatory sequence(s) are connected in such a way to control or permit gene expression when the appropriate molecular, e.g., transcriptional activator proteins, are bound to the regulatory sequence(s).



-36-

As used herein, production by recombinant means by using recombinant DNA methods means the use of the well known methods of molecular biology for expressing proteins encoded by cloned DNA, including cloning expression of genes and methods, such as gene  
5 shuffling and phage display with screening for desired specificities.

As used herein, a splice variant refers to a variant produced by differential processing of a primary transcript of genomic DNA that results in more than one type of mRNA.

As used herein, a composition refers to any mixture of two or more  
10 products or compounds. It can be a solution, a suspension, liquid; powder, a paste, aqueous, non-aqueous or any combination thereof.

As used herein, a combination refers to any association between two or more items.

As used herein, substantially identical to a product means  
15 sufficiently similar so that the property of interest is sufficiently unchanged so that the substantially identical product can be used in place of the product.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked.  
20 One type of exemplary vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Exemplary vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression  
25 vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. "Plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of

-37-

vector. Other such other forms of expression vectors that serve equivalent functions and that become known in the art subsequently hereto.

As used herein, vector also is used interchangeable with "virus  
5 vector" or "viral vector. In this case, which will be clear from the context, the "vector" is not self-replicating. Viral vectors are engineered viruses that are operatively linked to exogenous genes to transfer (as vehicles or shuttles) the exogenous genes into cells.

As used herein, transduction refers to the process of gene transfer  
10 into and expression in mammalian and other cells mediated by viruses. Transfection refers to the process when mediated by plasmids.

As used herein, transformation refers to the process of gene transfer into and expression in bacterial cells mediated by plasmids.

As used herein, "allele," which is used interchangeably herein with  
15 "allelic variant" refers to alternative forms of a gene or portions thereof. Alleles occupy the same locus or position on homologous chromosomes. When a subject has two identical alleles of a gene, the subject is said to be homozygous for the gene or allele. When a subject has two different alleles of a gene, the subject is said to be heterozygous for the gene.  
20 Alleles of a specific gene can differ from each other in a single nucleotide, or several nucleotides, and can include substitutions, deletions, and insertions of nucleotides. An allele of a gene also can be a form of a gene containing a mutation.

As used herein, the term "gene" or "recombinant gene" refers to a  
25 nucleic acid molecule comprising an open reading frame and including at least one exon and (optionally) an intron sequence. A gene can be either RNA or DNA. Genes can include regions preceding and following the coding region (leader and trailer).

-38-

As used herein, "intron" refers to a DNA sequence present in a given gene which is spliced out during mRNA maturation.

As used herein, "nucleotide sequence complementary to the nucleotide sequence set forth in SEQ ID NO:" refers to the nucleotide  
5 sequence of the complementary strand of a nucleic acid strand having the particular SEQ ID NO:. The term "complementary strand" is used herein interchangeably with the term "complement." The complement of a nucleic acid strand can be the complement of a coding strand or the complement of a non-coding strand. When referring to double stranded  
10 nucleic acids, the complement of a nucleic acid having a particular SEQ ID NO: refers to the complementary strand of the strand set forth in the particular SEQ ID NO: or to any nucleic acid having the nucleotide sequence of the complementary strand of the particular SEQ ID NO:.  
When referring to a single stranded nucleic acid having a nucleotide  
15 sequence corresponding to a particular SEQ ID NO:, the complement of this nucleic acid is a nucleic acid having a nucleotide sequence which is complementary to that of the particular SEQ ID NO:.

As used herein, the term "coding sequence" refers to that portion of a gene that encodes an amino acid sequence of a protein.

20 As used herein, the term "sense strand" refers to that strand of a double-stranded nucleic acid molecule that has the sequence of the mRNA that encodes the amino acid sequence encoded by the double-stranded nucleic acid molecule.

As used herein, the term "antisense strand" refers to that strand of  
25 a double-stranded nucleic acid molecule that is the complement of the sequence of the mRNA that encodes the amino acid sequence encoded by the double-stranded nucleic acid molecule.

As used herein, an array refers to a collection of elements, such as nucleic acid molecules, containing three or more members. An

-39-

addressable array is one in which the members of the array are identifiable, typically by position on a solid phase support or by virtue of an identifiable or detectable label, such as by color, fluorescence, electronic signal (*i.e.*, RF, microwave or other frequency that does not substantially alter the interaction of the molecules of interest), bar code or other symbology, chemical or other such label. In certain embodiments, the members of the array are immobilized to discrete identifiable loci on the surface of a solid phase or directly or indirectly linked to or otherwise associated with the identifiable label, such as affixed to a microsphere or other particulate support (herein referred to as beads) and suspended in solution or spread out on a surface.

As used herein, a support (also referred to as a matrix support, a matrix, an insoluble support or solid support) refers to any solid or semisolid or insoluble support to which a molecule of interest, typically a biological molecule, organic molecule or biospecific ligand is linked or contacted. Such materials include any materials that are used as affinity matrices or supports for chemical and biological molecule syntheses and analyses, such as, but are not limited to: polystyrene, polycarbonate, polypropylene, nylon, glass, dextran, chitin, sand, pumice, agarose, polysaccharides, dendrimers, buckyballs, polyacryl-amide, silicon, rubber, and other materials used as supports for solid phase syntheses, affinity separations and purifications, hybridization reactions, immunoassays and other such applications. The matrix herein can be particulate or can be in the form of a continuous surface, such as a microtiter dish or well, a glass slide, a silicon chip, a nitrocellulose sheet, nylon mesh, or other such materials. When particulate, typically the particles have at least one dimension in the 5-10 mm range or smaller. Such particles, referred collectively herein as "beads," are often, but not necessarily, spherical. Such reference, however, does not constrain the geometry of the matrix,

-40-

which can be any shape, including random shapes, needles, fibers, and elongated. Roughly spherical "beads," particularly microspheres that can be used in the liquid phase, also are contemplated. The "beads" can include additional components, such as magnetic or paramagnetic

- 5 particles (see, *e.g.*, Dynabeads (Dynal, Oslo, Norway)) for separation using magnets, as long as the additional components do not interfere with the methods and analyses herein.

- As used herein, a matrix or support particles refers to matrix materials that are in the form of discrete particles. The particles have any  
10 shape and dimensions, but typically have at least one dimension that is 100 mm or less, 50 mm or less, 10 mm or less, 1 mm or less, 100  $\mu\text{m}$  or less, 50  $\mu\text{m}$  or less and typically have a size that is 100  $\text{mm}^3$  or less, 50  $\text{mm}^3$  or less, 10  $\text{mm}^3$  or less, and 1  $\text{mm}^3$  or less, 100  $\mu\text{m}^3$  or less and can be order of cubic microns. Such particles are collectively called "beads."

- 15 As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, *Biochem.*, 11:942-944, 1972).

20 **B. Directed Evolution**

To date, there have been three general approaches described for protein directed evolution based on mutagenesis.

**1) Pure Random Mutagenesis**

- Random mutagenesis methodology requires that the amino acids in  
25 the starting protein sequence are replaced by all (or a group) of the 20 amino acids. Either single or multiple replacements at different amino acid positions are generated on the same molecule, at the same time. The random mutagenesis method relies on a direct search for fitness improvement based on random amino acid replacement and sequence

-41-

changes at multiple amino acid positions. In this approach neither the amino acid position (first dimension) nor the amino acid type (second dimension) are restricted; and everything possible is generated and tested. Multiple replacements can randomly happen at the same time on the same molecule. For example, random mutagenesis methods are widely used to develop antibodies with higher affinity for its ligand, by the generation of random-sequence libraries of antibody molecules, followed by expression and screening using filamentous phages.

## 2) Restricted Random Mutagenesis

- 10        Restricted random mutagenesis methods introduce either all of the 20 amino acids or DNA-biased residues, wherein the bias is based on the sequence of the DNA and not on that of the protein, in a stochastic or semi-stochastic manner, respectively, within restricted or predefined regions of the protein, known in advance to be involved in the biological activity being "evolved." This method relies on a direct search for fitness improvement based on random amino acid replacement and sequence changes at either restricted or multiple amino acid positions. In this approach the scanning can be restricted to selected amino acid positions and/or amino acid types, while material changes continue to be random in position and type. For example, the amino acid position can be restricted by prior selection of the target region to be mutated (selection of target region is based upon prior knowledge on protein structure/function); while the amino acid type is not primarily restricted as replacing amino acids are stochastically or at most "semi-stochastically" chosen. As an example, this method is used to optimize known binding sites on proteins, including hormone-receptor systems and antibody-epitope systems.
- 15
- 20
- 25

-42-

### 3) Non-restricted Rational mutagenesis

Rational mutagenesis is a two-step process and is described in co-pending U.S. application Serial No. 10/022,249. Briefly, the first step requires amino acid scanning where all and each of the amino acids in the

5 starting protein sequence are replaced by a third amino acid of reference (e.g., alanine). Only a single amino acid is replaced on each protein molecule at a time. A collection of protein molecules having a single amino acid replacement is generated such that molecules differ from each other by the amino acid position at which the replacement has taken

10 place. Mutant DNA molecules are designed, generated by mutagenesis and cloned individually, such as in addressable arrays, such that they are physically separated from each other and such that each one is the single product of an independent mutagenesis reaction. Mutant protein molecules derived from the collection of mutant nucleic acid molecules

15 also are physically separated from each other, such as by formatting in addressable arrays. Activity assessment on each protein molecule allows for the identification of those amino acid positions that result in a drop in activity when replaced, thus indicating the involvement of that particular amino acid position in the protein's biological activity and/or conformation

20 that leads to fitness of the particular feature being evolved. Those amino acid positions are referred to as HITs. At the second step, a new collection of molecules is generated such that each molecule differs from each of the others by the amino acid present at the individual HIT positions identified in step 1. All 20 amino acids (19 remaining) are

25 introduced at each of the HIT positions identified in step 1; while each individual molecule contains, in principle, one and only one amino acid replacement. Mutant DNA molecules are designed, generated by mutagenesis and cloned individually, such as in in addressable arrays, such that they are physically separated from each other and such that

-43-

each one is the single product of an independent mutagenesis reaction. Mutant protein molecules derived from the collection of mutant DNA molecules also are physically separated from each other, such as by formatting in addressable arrays. Activity assessment then is individually  
5 performed on each individual mutant molecule. The newly generated mutants that lead to a desired alteration (such as an improvement) in a protein activity are referred to as LEADs. This method permits an indirect search for activity alteration, such as improvement, based on one rational amino acid replacement and sequence change at a single amino acid  
10 position at a time, in search of a new, unpredicted amino acid sequence at some unpredicted regions along a protein to produce a protein that exhibits a desired activity or altered activity, such as better performance than the starting protein.

In this approach, neither the amino acid position nor the replacing  
15 amino acid type are restricted. Full length protein scanning is performed during the first step to identify HIT positions, and then all 20 amino acids are tested at each of the HIT positions, to identify LEAD sequences; while, as a starting point, only one amino acid at a time is replaced on each molecule. The selection of the target region (HITs and surrounding  
20 amino acids) for the second step is based upon experimental data on activity obtained in the first step. Thus, no prior knowledge of protein structure and/or function is necessary. Using this approach, LEAD sequences have been found on proteins that are located at regions of the protein not previously known to be involved in the particular biological  
25 activity being optimized; thus emphasizing the power of this approach to discover unpredictable regions (HITs) as targets for fitness improvement.

#### **C. 2-Dimensional Rational Scanning**

The 2-Dimensional rational scanning (or "2-dimensional scanning") methods for protein rational evolution provided herein (see, also



-44-

copending U.S. application Serial No. Attorney docket no. 923, filed the same day herewith, based on U.S. provisional application Serial Nos. 60/457,063 and 60/410,258) are based on scanning over two dimensions. The first dimension scanned is amino acid position along the

5 protein sequence to identify is-HIT target positions, and the second dimension is the amino acid type selected for replacing a particular is-HIT amino acid position. An advantage of the 2-dimensional scanning methods provided herein is that at least one, and typically both, of the amino acid position scan and/or the replacing amino acid scan can be

10 restricted such that fewer than all amino acids on the protein-backbone are selected for amino acid replacement; and/or fewer than all of the remaining 19 amino acids available to replace an original, such as native, amino acid are selected for replacement.

In particular embodiments, based on *i*) the particular protein

15 properties to be evolved, *ii*) the protein's amino acid sequence, and *iii*) the known properties of the individual amino acids, a number of target positions along the protein sequence are selected, *in silico*, as "is-HIT target positions." This number of is-HIT target positions is as large as possible such that all reasonably possible target positions for the

20 particular feature being evolved are included. In particular, embodiments where a restricted number of is-HIT target positions are selected for replacement, the amino acids selected to replace the is-HIT target positions on the particular protein being optimized can be either all of the remaining 19 amino acids or, more frequently, a more restricted group

25 comprising selected amino acids that are contemplated to have the desired effect on protein activity. In another embodiment, so long as a restricted number of replacing amino acids are used, all of the amino acid positions along the protein backbone can be selected as is-HIT target positions for amino acid replacement. Mutagenesis then is performed by

-45-

the replacement of single amino acid residues at specific is-HIT target positions on the protein backbone (e.g., "one-by-one," such as in addressable arrays), such that each individual mutant generated is the single product of each single mutagenesis reaction. Mutant DNA

5 molecules are designed, generated by mutagenesis and cloned individually, such as in addressable arrays, such that they are physically separated from each other and that each one is the single product of an independent mutagenesis reaction. Mutant protein molecules derived from the collection of mutant DNA molecules also are physically separated

10 from each other, such as by formatting in in addressable arrays. Thus, a plurality of mutant protein molecules are produced. Each mutant protein contains a single amino acid replacement at only one of the is-HIT target positions. Activity assessment is then individually performed on each individual protein mutant molecule, following protein expression and

15 measurement of the appropriate activity. An example of practice of this this method is shown in the Example in which mutant IFN $\alpha$  molecules and IFN $\beta$  molecules are produced.

The newly generated proteins that lead to altered, typically improvement, in a target protein activity are referred to as LEADs. This

20 method relies on an indirect search for protein improvement for a particular activity, such as increased resistance to proteolysis, based on a rational amino acid replacement and sequence change at single or, in another embodiment, a limited number of amino acid positions at a time. As a result, optimized proteins that have new amino acid sequences at

25 some regions along the protein that perform better (at a particular target activity or other property) than the starting protein are identified and isolated.

-46-

### 1) Identifying *in-silico* HITs

Provided herein is a method for directed evolution that includes identifying and selecting (using *in silico* analysis) specific amino acids and amino acid positions (referred to herein as is-HITs) along the protein  
5 sequence that are contemplated to be directly or indirectly involved in the feature being evolved. As noted, the 2-dimensional scanning methods provided include the following two-steps. The first step is an *in silico* search of a target protein's amino acid sequence to identify all possible amino acid positions that potentially can be targets for the activity being  
10 evolved. This is effected, for example, by assessing the effect of amino acid residues on the property(ies) to be altered on the protein, using any known standard software. The particulars of the *in silico* analysis is a function of the property to be modified. For example, in the example herein, a property that is altered resistance of the protein to proteolysis..  
15 To determine aminoacid residues that are potential targets as is-HITs, in this example, all possible target residues for proteases were first identified. The 3-dimensional structure of the protein was then considered in order to identify surface residues. Comparison of exposed residues with proteolytically cleavable residues yields residues that are targets for  
20 change.

Once identified, these amino acid positions or target sequences are referred to as "is-HITs" (*in silico* HITs). *In silico* HITs are defined as those amino acid positions (or target positions) that potentially are involved in the "evolving" feature, such as increased resistance to proteolysis. In  
25 one embodiment, the discrimination of the is-HITs among all the amino acid positions in a protein sequence is made based on i) the amino acid type at each position in addition to, whenever available but not necessarily, ii) the information on the protein secondary or tertiary structure. *In silico* HITs constitute a collection of mutant molecules such

-47-

that all possible amino acids, amino acid positions or target sequences potentially involved in the evolving feature are represented. No strong theoretical discrimination among amino acids or amino acid positions is made at this stage.

- 5           *In silico* HIT positions are spread over the full length of the protein sequence. In one embodiment, only a single is-HIT amino acid at a time is replaced on the target protein. In another embodiment, a limited number of is-HIT amino acids are replaced at the same time on the same target protein molecule. The selection of target regions (is-HITs and
- 10   surrounding amino acids) for the second step is based upon rational assumptions and predictions. No prior knowledge of protein structure/function is necessary. Hence, the 2-dimensional scanning methodology provided herein does not require any previous knowledge of the 3-dimensional conformational structure of the protein.
- 15           Any protein known or otherwise available to those of skill in the art is suitable for optimization using the directed evolution methods provided herein, including cytokines (e.g., IFN $\alpha$ -2b) or any other proteins that have previously been mutated or optimized.

- 20           A variety of parameters can be analyzed to determine whether or not a particular amino acid on a protein might be involved in the evolving feature. For example, the information provided by crystal structures of proteins can be rationally exploited in order to perform a computer-assisted (*in silico*) analysis towards the prediction of variants with desired features. In a particular embodiment, a limited number of initial premises
- 25   (typically no more than 2) are used to determine the *in silico* HITs. In other embodiments, the number of premises used to determine the *in silico* hits can range from 1 to 10 premises, including no more than 9, no more than 8, no more than 7, no more than 6, no more than 5, no more than 4, no more than 3, but are typically no more than 2 premises. It is

-48-

important to the methods provided herein that the number of initial premises be kept to a minimum, so as to maintain the number of potential is-HITs at a maximum (here is where the methods provided are not limited by too much prediction based on theoretical assumptions). When two  
5 premises are employed, the first condition is typically the amino acid type itself, which is directly linked to the nature of the evolving feature. For example, if the goal were to change the optimum pH for an enzyme, then the replacing amino acids selected at this step for the replacement of the original sequence would be only those with a certain pKa value. The  
10 second premise is typically related to the specific position of those amino acids along the protein structure. For example, some amino acids might be discarded if they are not expected to be exposed enough to the solvent, even when they might have appropriate pKa values.

During the first step of identification of is-HITs according to the  
15 methods provided herein, each individual amino acid along the protein sequence is considered individually to assess whether it is a candidate for is-HIT. This search is done one-by-one and the decision on whether the amino acid is considered to be a candidate for a is-HIT is based on (1) the amino acid type itself; (2) the position on the amino acid sequence and  
20 protein structure if known; and (3) the predicted interaction between that amino acid and its neighbors in sequence and space.

Using the 3D-scanning methods provided herein, once one protein within a family of proteins (e.g., IFN $\alpha$ -2b within the cytokine family) is optimized using the methods provided herein for generating LEAD  
25 mutants, is-HITs can be identified on other or all proteins within a particular family by identifying the corresponding amino acid positions therein using structural homology analysis (based upon comparisons of the 3-D structures of the family members with original protein to identify corresponding residues for replacement) as described hereinafter. The is-

-49-

HITs on family identified in this manner then can be subjected to the next step of identifying replacing amino acids and further assayed to obtain LEADs or super-LEADs as described herein.

## 2) Identifying Replacing Amino Acids

- 5        Once the is-HITs target positions are selected, the next step is identifying those amino acids that will replace the original, such as native, amino acid at each is-HIT position to alter the activity level for the particular feature being evolved. The set of replacing amino acids to be used to replace the original, such as native, amino acid at each is-HIT
- 10   position can be different and specific for the particular is-HIT position. The choice of the replacing amino acids takes into account the need to preserve the physicochemical properties such as hydrophobicity, charge and polarity, of essential (e.g., catalytic, binding, etc.) residues. The number of replacing amino acids, of the remaining 19 non-native (or non-
- 15   original) amino acids, that can be used to replace a particular is-HIT target position ranges from 1 up to about 19, from 1 up to about 15, from 1 up to about 10, from 1 up to about 9, from 1 up to about 8, from 1 up to about 7, from 1 up to about 6, from 1 up to about 5, from 1 up to about 4, from 1 up to about 3, or from 1 to 2 amino acid replacements.
- 20        Numerous methods of selecting replacing amino acids (also referred to herein as "replacement amino acids") are well known in the art. Protein chemists determined that certain amino acid substitutions commonly occur in related proteins from different species. As the protein still functions with these substitutions, the substituted amino acids are
- 25   compatible with protein structure and function. Often, these substitutions are to a chemically similar amino acid, but other types of changes, although relatively rare, can also occur.

Knowing the types of changes that are most and least common in a large number of proteins can assist with predicting alignments and amino

-50-

acid substitutions for any set of protein sequences. Amino acid substitution matrices are used for this purpose.

In amino acid substitution matrices, amino acids are listed across the top of a matrix and down the side, and each matrix position is filled with a score that reflects how often one amino acid would have been paired with the other in an alignment of related protein sequences. The probability of changing amino acid A into amino acid B is assumed to be identical to the reverse probability of changing B into A. This assumption is made because, for any two sequences, the ancestor amino acid in the phylogenetic tree is usually not known. Additionally, the likelihood of replacement should depend on the product of the frequency of occurrence of the two amino acids and on their chemical and physical similarities. A prediction of this model is that amino acid frequencies will not change over evolutionary time (Dayhoff *et al.*, *Atlas of Protein Sequence and Structure*, 5(3):345-352, 1978). Below are several exemplary amino acid substitution matrices, including, but not limited to block substitution matrix (BLOSUM), Jones, Gonnet, Fitch, Feng, McLachlan, Grantham, Miyata, Rao, Risler, Johnson and percent accepted mutation (PAM). Any such method known to those of skill in the art can be employed.

**(a) Percent accepted mutation (PAM)**

Dayhoff and coworkers developed a model of protein evolution that resulted in the development of a set of widely used replacement matrices (Dayhoff *et al.*, *Atlas of Protein Sequence and Structure*, 5(3):345-352, 1978) termed percent accepted mutation matrices (PAM). In deriving these matrices, each change in the current amino acid at a particular site is assumed to be independent of previous mutational events at that site. Thus, the probability of change of any amino acid A to amino acid B is the same, regardless of the previous changes at that site and also regardless of the position of amino acid A in a protein sequence.

-51-

In the Dayhoff approach, replacement rates are derived from alignments of protein sequences that are at least 85% identical; this constraint ensures that the likelihood of a particular mutation being the result of a set of successive mutations is low. Because these changes  
5 are observed in closely related proteins, they represent amino acid substitutions that do not significantly change the function of the protein. Hence, they are called "accepted mutations," as defined as amino acid changes that are accepted by natural selection.

(i) PAM Analysis

10 In particular embodiments of the methods provided herein, "Percent Accepted Mutation" (PAM; Dayhoff *et al.*, *Atlas of Protein Sequence and Structure*, 5(3):345-352, 1978 FIG2) PAM values are used to select an appropriate group of replacement amino acids. PAM matrices were originally developed to produce alignments between protein sequences  
15 based evolutionary distances. Because, in a family of proteins or homologous (related) sequences, identical or similar amino acids (85% similarity) are shared, conservative substitutions for, or allowed point mutations of the corresponding amino acid residues can be determined throughout an aligned reference sequence. In this regard, "conservative  
20 substitutions" of a residue in a reference sequence are those substitutions that are physically and functionally similar to the corresponding reference residues, e.g., that have a similar size, shape, electric charge, chemical properties, including the ability to form covalent or hydrogen bonds, or the like. Particularly suitable conservative amino acid substitutions are  
25 those that show the highest scores and fulfill the PAM matrix criteria in the form of "accepted point mutations." For example, by comparing a family of scoring matrices, Dayhoff *et al.*, *Atlas of Protein Sequence and Structure*, 5(3):345-352, 1978, found a consistently higher score



-52-

significance when using PAM250 matrix to analyze a variety of proteins, known to be distantly related.

(ii) PAM 250

In a particular embodiment, the PAM250 matrix set forth in FIG2 is  
5 used for determining the replacing amino acids based on similarity criteria. The PAM250 matrix uses data obtained directly from natural evolution to facilitate the selection of replacing amino acids for the is-HITs to generate conservative mutations without much affecting the overall protein  
10 function. By using the PAM250 matrix, candidate replacing amino acids are identified from related proteins from different organisms.

(b) Jones and Gonnet

This method (see, *e.g.*, Jones *et al.*, *Comput. Appl. Biosci.*, 8:275-282, 1992 and Gonnet *et al.*, *Science*, 256:1433-1445, 1992) uses  
15 much of the same methodology as Dayhoff (see below), but with modern databases. The matrix of Jones *et al.*, is extracted from Release 15.0 of the SWISS-PROT protein sequence database. Point mutations totaling 59,160 from 16,130 protein sequences were used to calculate a PAM250 (see below) matrix.

The matrix published by Gonnet *et al.*, *Science*, 256:1433-1445,  
20 1992, was built from a sequence database of 8,344,353 amino acid residues. Each sequence was compared against the entire database, such that  $1.7 \times 10^6$  subsequent matches resulted for the significant alignments. These matches were then used to generate a matrix with a PAM distance of 250.

25 (c) Fitch and Feng

Fitch, *J. Mol. Evol.*, 16(1):9-16, 1966, used an exchange matrix that contained for each pair (A, B) of amino acid types the minimum number of nucleotides that must be changed to encode amino acid A instead of amino acid B. Feng *et al.*, *J. Mol. Evol.*, 21:112-125, 1985,

-53-

used an enhanced version of Fitch, *J. Mol. Evol.*, 16(1):9-16, 1966, to build a Structure-Genetic matrix. In addition to considering the minimum number of base changes required to encode amino acid B instead of A, this method also considers the structural similarity of the amino acids.

5                   (d)     **McLachlan, Grantham and Miyata**

McLachlan, *J. Mol. Biol.*, 61:409-424 1971, used 16 protein families, each with 2 to 14 members. The 89 sequences were aligned and the pairwise exchange frequency, observed in 9280 substitutions, was used to generate an exchange matrix with values varying from 0  
10 to 9.

Grantham, *Science*, 185:862-864, 1974, considers composition, polarity and molecular volume of amino acid side-chains, properties that were highly correlated to the relative substitution frequencies tabulated by McLachlan, *J. Mol. Biol.*, 61:409-424, 1971, to build the matrix.

15                   Miyata, *J. Mol. Evol.*, 12:219-236, 1979, uses the volume and polarity values of amino acids published by Grantham, *Science*, 185:862-864, 1974. For every amino acid type pair, the difference for both properties was calculated and divided by the standard deviation of all the differences. The square root of the sum of both values is then used in  
20 the matrix.

(e)     **Rao**

Rao, *J. Pept. Protein Res.*, 29:276-281, 1987, employs five amino acid properties to create a matrix; namely, alpha-helical, beta-strand and reverse-turn propensities as well as polarity and hydrophobicity. The  
25 standardized properties were summed and the matrix rescaled to the same average as that for PAM (Dayhoff *et al.*, *Atlas of Protein Sequence and Structure*, 5(3):345-352, 1978).

(f)     **Risler**

-54-

Risler *et al.*, *J. Mol. Biol.*, 204:1019-1029, 1988, aligned 32 three-dimensional structures from 11 protein families by rigid-body superposition of the backbone topology. Only substitutions were considered where at least three adjacent and equivalent main-chain C $\alpha$  atom pairs in the compared structures were each not more than 1.2 Å apart. A total of 2860 substitutions were considered and used to build a matrix based on  $\chi^2$  distance calculations.

(g) Johnson

Johnson *et al.*, *J. Mol. Biol.*, 233:716-738, 1993, derived their matrix from the tertiary structural alignment of 65 families in a database of 235 structures created with the method of Sali *et al.*, *J. Mol. Biol.*, 212:403-428, 1990. Their examination of the substitutions was based on the expected and observed ratios of occurrences and the final matrix values were taken as  $\log_{10}$  of the ratios.

15 (h) Block Substitution Matrix (BLOSUM)

One empirical approach (Henikoff *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:10915-10919, 1992) uses local, ungapped alignments of distantly related sequences to derive the blocks amino acid substitution matrix (BLOSUM) series of matrices. The matrix values are based on the observed amino acid substitutions in a larger set of about 2000 conserved amino acid patterns, termed blocks. These blocks act as signatures of families of related proteins. Matrices of this series are identified by a number after the matrix (e.g., BLOSUM50), which refers to the minimum percentage identity of the blocks of multiple aligned amino acids used to construct the matrix. It is noteworthy that these matrices are directly calculated without extrapolations, and are analogous to transition probability matrices  $P(T)$  for different values of  $T$ , estimated without reference to any rate matrix  $Q$ .

-55-

The outcome of these two steps set forth above, which is performed *in silico* is that: (1) the amino acid positions that will be the target for mutagenesis are identified; these positions are referred to as is-HITs; (2) the replacing amino acids for the original, such as native, amino acids at the is-HITs are identified, to provide a collection of candidate LEAD mutant molecules that are expected to perform different from the native one. These are are assayed for a desired optimized (or improved or altered) biological activity.

10           **3)     Physical Construction of Mutant Proteins and Biological Assays**

Once is-HITs are selected as set forth above, replacing amino acids are introduced. Mutant proteins typically are prepared using recombinant DNA methods and assessed in appropriate biological assays for the particular biological activity (feature) optimized (see, *e.g.*, Example 1).

15   An exemplary method of preparing the mutant proteins is by mutagenesis of the original, such as native, gene using methods well known in the art. Mutant molecules are generated one-by-one, such as in addressable arrays, such that each individual mutant generated is the single product of each single and independent mutagenesis reaction. Individual mutagenesis

20   reactions are conducted separately, such as in addressable arrays where they are physically separated from each other. Once a population of sets of nucleic acid molecules encoding the respective mutant proteins is prepared, each is separately introduced one-by-one into appropriate cells for the production of the corresponding mutant proteins. This can also be

25   performed, for example, in addressable arrays where each set of nucleic acid molecules encoding a respective mutant protein is introduced into cells confined to a discrete location, such as in a well of a multi-well microtiter plate. Each individual mutant protein is individually phenotypically characterized and performance is quantitatively assessed

-56-

using assays appropriate for the feature being optimized (i.e., feature being evolved). Again, this step can be performed in addressable arrays. Those mutants displaying a desired increased or decreased performance compared to the original, such as native molecules are identified and

5 designated LEADs. From the beginning of the process of generating the mutant DNA molecules up through the readout and analysis of the performance results, each candidate LEAD mutant is generated, produced and analyzed individually, such as from its own address in an addressable array. The process is amenable to automation.

10 D. 2-Dimensional Scanning of Proteins for Increased Resistance to Proteolysis

The methods of 2-dimensional scanning permit preparation of proteins modified for a selected trait, activity or other phenotype. Among modifications of interest for therapeutic proteins are those that increase

15 protection against protease digestion while maintaining the requisite biological activity. Such changes are useful for producing longer-lasting therapeutic proteins.

The delivery of stable peptide and protein drugs to patients is a major challenge for the pharmaceutical industry. These types of drugs in

20 the human body are constantly eliminated or taken out of circulation by different physiological processes including internalization, glomerular filtration and proteolysis. The latter is often the limiting process affecting the half-life of proteins used as therapeutic agents in per-oral administration and either intravenous or intramuscular injections.

25 The 2-dimensional scanning process for protein evolution is used to effectively improve protein resistance to proteases and thus increase protein half-life *in vitro* and, ultimately *in vivo*. As noted, the methods provided herein for designing and generating highly stable, longer lasting proteins, or proteins having a longer half-life include: 1) identifying some

-57-

or all possible target sites on the protein sequence that are susceptible to digestion by one or more specific proteases (these sites are referred to herein as is-HITs); *ii*) identifying appropriate replacing amino acids, specific for each is-HIT, such that upon replacement of one or more of the original, such as native, amino acids at that specific is-HIT, they can be expected to increase the is-HIT's resistance to digestion by protease while at the same time, maintaining or improving the requisite biological activity of the protein (these proteins with replaced amino acids are the "candidate LEADs"); *iii*) systematically introducing the specific replacing amino acids (candidate LEADs) at every specific is-HIT target position to generate a collection containing the corresponding mutant candidate lead molecules. Mutants are generated, produced and phenotypically characterized one-by-one, such as in addressable arrays, such that each mutant molecule contains initially an amino acid replacement at only one is-HIT site.

In particular embodiments, such as in subsequent rounds, mutant molecules also can be generated that contain one or more amino acids at one or more is-HIT sites that have been replaced by candidate LEAD amino acids. Those mutant proteins carrying one or more mutations at one or more is-HITs, and that display improved protease resistance are called LEADs (one mutation at one is-HIT) and super-LEADs (mutations at more than one is-HIT).

The first step of the process takes into consideration existing knowledge from different domains:

(1) About the galenic and the delivery environment (tissue, organ or corporal fluid) of the particular therapeutic protein in order to establish a list of proteases more likely to be found in that environment. For example, a therapeutic protein in per-oral application is likely to encounter typical proteases of the luminal gastrointestinal tract. In

-58-

contrast, if this protein were injected in the blood circulation, serum proteases would be implicated in the proteolysis. Based on the specific list of proteases involved, the complete list of all amino acid sequences that potentially could be targeted by the proteases in the list is

5 determined.

(2) Since protease mixtures in the body are quite complex in composition, almost all the residues in any target protein potentially are targeted for proteolysis (FIG6A). Nevertheless, proteins form specific tri-dimensional structures where residues are more or less exposed to the  
 10 environment and protease action. It can be assumed that those residues constituting the core of a protein are inaccessible to proteases, while those more 'exposed' to the environment are better targets for proteases. The probability for every specific amino acid to be 'exposed' and then to be accessible to proteases can be taken into account to reduce the  
 15 number of is-HIT. Consequently, the methods herein consider the analysis with respect to solvent "exposure" or "accessibility" for each individual amino acid in the protein sequence. Solvent accessibility of residues can alternatively be estimated, regardless of any previous knowledge of specific protein structural data, by using an algorithm  
 20 derived from empirical amino acid probabilities of accessibility, which is expressed in the following equation (Boger *et al.*, *Reports of the Sixth International Congress in Immunology*, p. 250, 1986):

25 
$$A(i) = \left[ \prod_{j=1}^6 \delta_{-i+4-j} \right] \cdot [0.62]^{-6}.$$

Briefly, these are fractional probabilities ( $\delta_{-i}$ ) determined for an amino acid (i) found on the surface of a protein, which are based upon structural data from a set of several proteins. It is thus possible to calculate the solvent accessibility (A) of an amino acid (A(i)) at sequence

-59-

position (i-2 to i+3, onto a sliding window of length equal to 6) that is within an average surface accessible to solvent of >20 square angstroms ( $\text{\AA}^2$ ).

The protease accessible target amino acids along the protein  
5 sequence, i.e., the amino acids to be replaced, are thus identified and are referred to herein as *in silico* HITs (is-HITs).

Amino acids at the is-HITs then are replaced by residues that render the sequence less vulnerable (by a factor, for example, of 1%, 10%, 20%, 30%, 40%, 50%, . . . 100% depending upon the protein) or  
10 invulnerable (substantially no detectable digestion within a set time period) to protease digestion, while at the same time maintain a biological activity or activities of interest of the protein. The choice of the replacing amino acids is complicated by (1) the broad target specificity of certain proteases and (2) the need to preserve the physicochemical properties  
15 such as hydrophobicity, charge and polarity, of essential (e.g., catalytic, binding and/or other activities depending upon the protein) residues. For use in the methods herein, the "Percent Accepted Mutation" values (PAM values; see, Dayhoff *et al.*, *Atlas of Protein Sequence and Structure*, 5(3):345-352, 1978), FIG2) can be used as described herein. PAM values,  
20 originally developed to produce alignments between protein sequences, are available in the form of probability matrices, which reflect an evolutionary distance. Since, in a family of proteins or homologous (related) sequences, identical or similar amino acids (85% similarity) are shared, conservative substitutions for, or "allowed point mutations" of  
25 the corresponding amino acid residues can be determined throughout an aligned reference sequence. In this regard, "conservative substitutions" of a residue in a reference sequence are those substitutions that are physically and functionally similar to the corresponding reference residues, e.g., that have a similar size, shape, electric charge, chemical



-60-

properties, including the ability to form covalent or hydrogen bonds, or the like. For example, conservative substitutions can be those that exhibit the highest scores and fulfill the PAM matrix criteria in the form of "accepted point mutations."

- 5 By comparing a family of scoring matrices, Dayhoff *et al.*, *Atlas of Protein Sequence and Structure*, 5(3):345-352, 1978), found consistently higher score significance when using PAM250 matrix to analyze a variety of proteins, known to be distantly related. For methods herein, the PAM250 matrix was selected for use. The PAM250 matrix is used, by  
10 learning directly from natural evolution, to find replacing amino acids for the is-HITs to generate conservative mutations without affecting the protein function. By using PAM250, candidate replacing amino acids are identified from related proteins from different organisms.

- An exemplary class of proteins that can be optimized according to  
15 the methods provided herein are the cytokines. For example, 2D-scanning methods provided herein can be used to modify the following cytokines to increase their stability as assessed by an increased resistance to proteolysis resulting in an increased protein half-life in the bloodstream or any other desired biological activity of the selected  
20 protein. Exemplary cytokines, include, but are not limited to: interleukin-10 (IL-10; SEQ ID NO: 200), interferon beta (IFN $\beta$ ; SEQ ID NO: 196), interferon alpha-2a (IFN $\alpha$ -2a; SEQ ID NO: 182), interferon alpha-2b (IFN $\alpha$ -2b; SEQ ID NO: 1), and interferon gamma (IFN- $\gamma$ ; SEQ ID NO: 199), granulocyte colony stimulating factor (G-CSF; SEQ ID NO: 210), leukemia  
25 inhibitory factor (LIF; SEQ ID NO: 213), growth hormone (hGH; SEQ ID NO: 216), ciliary neurotrophic factor (CNTF; SEQ ID NO: 212), leptin (SEQ ID NO: 211), oncostatin M (SEQ ID NO: 214), interleukin-6 (IL-6; SEQ ID NO: 217), interleukin-12 (IL-12; SEQ ID NO: 215), erythropoietin (EPO; SEQ ID NO: 201), granulocyte-macrophage colony stimulating

-61-

factor (GM-CSF; SEQ ID NO: 202), interleukin-2 (IL-2; SEQ ID NO: 204),  
interleukin-3 (IL-3; SEQ ID NO: 205), interleukin-4 (IL-4; SEQ ID NO:  
207), interleukin-5 (IL-5; SEQ ID NO: 208), interleukin-13 (IL-13; SEQ ID  
NO: 209), Flt3 ligand (SEQ ID NO: 203) and stem cell factor (SCF; SEQ  
5 ID NO: 206).

Accordingly, provided herein are modified cytokines that exhibit  
increased resistance to proteolysis compared to the unmodified cytokine.  
The modified cytokines can be selected from the group consisting of a  
member of the interferons/interleukin-10 protein family, a member of the  
10 long-chain cytokine family; and a member of the short-chain cytokine  
family. In particular embodiments, the modified cytokines provided herein  
are selected from the group consisting of: interleukin-10 (IL-10),  
interferon beta (IFN $\beta$ ), interferon alpha-2a (IFN $\alpha$ -2a), interferon alpha-2b  
(IFN $\alpha$ -2b), and interferon gamma (IFN- $\gamma$ ), granulocyte colony stimulating  
15 factor (G-CSF), leukemia inhibitory factor (LIF), human growth hormone  
(hGH), ciliary neurotrophic factor (CNTF), leptin, oncostatin M, interleukin-  
6 (IL-6) and interleukin-12 (IL-12), erythropoietin (EPO), granulocyte-  
macrophage colony stimulating factor (GM-CSF), interleukin-2 (IL-2),  
interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-13  
20 (IL-13), Flt3 ligand and stem cell factor (SCF). In one embodiment, the  
modified cytokine is an interferon, including modified interferon  $\alpha$ -2b  
(IFN $\alpha$ -2b).

**E. Rational Evolution of IFN $\alpha$ -2b For Increased Resistance to  
Proteolysis**

25 IFN $\alpha$ -2b is used for a variety of applications. Typically it is used for  
treatment of type B and C chronic hepatitis. Additional indications  
include, but are not limited to, melanomas, herpes infections, Kaposi  
sarcomas and some leukemia and lymphoma cases. Patients receiving  
interferon are subject to frequent repeat applications of the drug. Since

-62-

such frequent injections generate uncomfortable physiological as well as undesirable psychological reactions in patients, increasing the half-life of interferons and thus decreasing the necessary frequency of interferon injections, would be extremely useful to the medical community. For

5 example, after injection of native human IFN $\alpha$ -2b injection in mice, as a model system, its presence can be detected in the serum between 3 and 10 hours with a half-life of only around 4 hours. The IFN $\alpha$ -2b completely disappears to undetectable levels by 18-24 hours after injection. Provided herein are mutant variants of the IFN $\alpha$ -2b protein that display altered

10 properties including: (a) highly improved stability as assessed by resistance to proteases in vitro and by pharmacokinetics studies in mice; and (b) at least comparable biological activity as assessed by antiviral and antiproliferative action compared to both the unmodified and wild type native IFN $\alpha$ -2b protein and to at least one pegylated derivative of the wild

15 type native IFN $\alpha$ . As a result, the IFN $\alpha$ -2b mutant proteins provided herein confer a higher half-life and at least comparable antiviral and antiproliferation activity (sufficient for a therapeutic effect) with respect to the native sequence and to the pegylated derivatives molecules currently being used for the clinical treatment of hepatitis C infection. See Figures

20 6(A)-6(N), 6(T) and 6(U). Thus, the optimized IFN $\alpha$ -2b protein mutants that possess increased resistance to proteolysis and/or glomerular filtration provided herein result in a decrease in the frequency of injections needed to maintain a sufficient drug level in serum, leading to i) higher comfort and acceptance by patients, ii) lower doses necessary to achieve

25 comparable biological effects, and iii) as a consequence of (ii), an attenuation of the (dose-dependent) secondary effects observed in humans.

In particular embodiments, the half-life of the IFN $\alpha$ -2b and IFN $\alpha$ -2a mutants provided herein is increased by an amount selected from at least

-63-

10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 150%, at least 200%, at least 250%, at least 300%, at least 350%, at least 400%, at least 450%, at least 500% or more, when compared to  
5 the half-life of native human IFN $\alpha$ -2b and IFN $\alpha$ -2a in either human blood, human serum or an in vitro mixture containing one or more proteases. In other embodiments, the half-life of the IFN $\alpha$ -2b and IFN $\alpha$ -2a mutants provided herein is increased by an amount selected from at least 6 times, 7 times, 8 times, 9 times, 10 times, 20 times, 30 times, 40 times, 50  
10 times, 60 times, 70 times, 80 times, 90 times, 100 times, 200 times, 300 times, 400 times, 500 times, 600 times, 700 times, 800 times, 900 times, 1000 times, or more, when compared to the half-life of native human IFN $\alpha$ -2b and IFN $\alpha$ -2a in either human blood, human serum or an in vitro mixture containing one or more proteases.

15 Two methodologies were used herein to increase the stability of IFN $\alpha$ -2b by amino acid replacement: *i*) amino acid replacement that leads to higher resistance to proteases by direct destruction of the protease target residue or sequence, while either maintaining or improving the requisite biological activity (e.g., antiviral activity, antiproliferation  
20 activity), and/or *ii*) amino acid replacement that leads to a different pattern of *N*-glycosylation, thus decreasing both glomerular filtration and sensitivity to proteases, while either improving or maintaining the requisite biological activity (e.g., antiviral activity, antiproliferation activity).

25 The 2D-scanning methods provided herein were used to identify the amino acid changes on IFN $\alpha$ -2b that lead to an increase in stability when challenged either with proteases, human blood lysate or human serum. Increasing protein stability to proteases, human blood lysate or human serum, and/or increasing the molecular size is contemplated herein to

-64-

provide a longer *in vivo* half-life for the particular protein molecules, and thus to a reduction in the frequency of necessary injections into patients. The biological activities that were measured for the IFN $\alpha$ -2b molecules are  
i) their capacity to inhibit virus replication when added to permissive cells  
5 previously infected with the appropriate virus, and ii) their capacity to stimulate cell proliferation when added to the appropriate cells. Prior to the measurement of biological activity, IFN $\alpha$ -2b molecules were challenged with proteases, human blood lysate or human serum during different incubation times. The biological activity measured, corresponds  
10 then to the residual biological activity following exposure to the protease-containing mixtures.

As set forth above, provided herein are methods for the development of IFN $\alpha$ -2b and IFN $\alpha$ -2a molecules that, while maintaining the requisite biological activity intact, have been rendered less susceptible  
15 to digestion by blood proteases and therefore display a longer half-life in blood circulation. In this particular example, the method used included the following specific steps as set forth in Example 2:

- 1) Identifying some or all possible target sites on the protein sequence that are susceptible to digestion by one or more specific  
20 proteases (these sites are the is-HITs) and
- 2) Identifying appropriate replacing amino acids, specific for each is-HIT, such that if used to replace one or more of the original amino acids at that specific is-HIT, they can be expected to increase the is-HIT's resistance to digestion by protease while at  
25 the same time, keeping the biological activity of the protein unchanged (these replacing amino acids are the "candidate LEADs").

As set forth in Example 2, the 3-dimensional structure of IFN $\alpha$ -2b obtained from the NMR structure of IFN $\alpha$ -2a (PDB code 1ITF) was used to

-65-

select only those residues exposed to solvent from a list of residues along the IFN $\alpha$ -2b and IFN $\alpha$ -2a sequence which can be recognized as a substrate for different enzymes present in the serum. Residue 1 corresponds to the first residue of the mature peptide IFN $\alpha$ -2b encoded by nucleotides

5 580-1074 of sequence accession No. J00207, SEQ ID NO:1. Using this approach, the following 42 amino acid target positions were identified as is-HITs on IFN $\alpha$ -2b or IFN $\alpha$ -2a, which numbering is that of the mature protein (SEQ ID NO:1 or SEQ ID NO:182, respectively): L3, P4, R12, R13, M16, R22, K23 or R23, F27, L30, K31, R33, E41, K49, E58, K70, E78,

10 K83, Y89, E96, E107, P109, L110, M111, E113, L117, R120, K121, R125, L128, K131, E132, K133, K134, Y135, P137, M148, R149, E159, L161, R162, K164, and E165. Each of these positions was replaced by residues defined as compatible by the substitution matrix PAM250 while at the same time not generating any new substrates for

15 proteases. For these 42 is-HITs, the residue substitutions determined by PAM250 analysis were as follows:

R to H, Q  
 E to H, Q  
 K to Q, T  
 20 L to V, I  
 M to I, V  
 P to A, S  
 Y to I, H.

25 **1) Modified IFN $\alpha$ -2b Proteins with Single Amino Acid Substitutions**

Among the mutant proteins provided herein, are mutant IFN $\alpha$ -2b proteins that have increased resistance proteolysis compared to the unmodified, typically wild-type, protein. The mutant IFN $\alpha$ -2b proteins include those selected from among proteins containing more single amino

-66-

acid replacements in SEQ ID NO:1, corresponding to: L by V at position 3; L by I at position 3; P by S at position 4; P by A at position 4; R by H at position 12; R by Q at position 12; R by H at position 13; R by Q at position 13; M by V at position 16; M by I at position 16; R by H at position 22; R by Q at position 22; R by H at position 23; R by Q at position 23; F by I at position 27; F by V at position 27; L by V at position 30; L by I at position 30; K by Q at position 31; K by T at position 31; R by H at position 33; R by Q at position 33; E by Q at position 41; E by H at position 41; K by Q at position 49; K by T at position 49; E by Q at position 58; E by H at position 58; K by Q at position 70; K by T at position 70; E by Q at position 78; E by H at position 78; K by Q at position 83; K by T at position 83; Y by H at position 89; Y by I at position 89; E by Q at position 96; E by H at position 96; E by Q at position 107; E by H at position 107; P by S at position 109; P by A at position 109; L by V at position 110; L by I at position 110; M by V at position 111; M by I at position 111; E by Q at position 113; E by H at position 113; L by V at position 117; L by I at position 117; R by H at position 120; R by Q at position 120; K by Q at position 121; K by T at position 121; R by H at position 125; R by Q at position 125; L by V at position 128; L by I at position 128; K by Q at position 131; K by T at position 131; E by Q at position 132; E by H at position 132; K by Q at position 133; K by T at position 133; K by Q at position 134; K by T at position 134; Y by H at position 135; Y by I at position 135; P by S at position 137; P by A at position 137; M by V at position 148; M by I at position 148; R by H at position 149; R by Q at position 149; E by Q at position 159; E by H at position 159; L by V at position 161; L by I at position 161; R by H at position 162; R by Q at position 162; K by Q at position 164; K by T at position 164; E by Q at position 165; and E by H at position 165.

-67-

## 2) LEAD Identification

Next the specific replacing amino acids (candidate LEADs) are systematically introduced at every specific is-HIT position to generate a collection containing the corresponding mutant IFN $\alpha$ -2b DNA molecules, as set forth in Example 2. The mutant DNA molecules were used to produce the corresponding mutant IFN $\alpha$ -2b protein molecules by transformation or transfection into the appropriate cells. These protein mutants were assayed for (i) protection against proteolysis, (ii) antiviral and antiproliferation activity in vitro, (iii) pharmacokinetics in mice. Of particular interest are mutations that increase these activities of the IFN $\alpha$ -2b mutant proteins compared to unmodified wild type IFN $\alpha$ -2b protein and to pegylated derivatives of the wild type protein. Based on the results obtained from these assays, each individual IFN $\alpha$ -2b variant was assigned a specific activity. Those variant proteins displaying the highest stability and/or resistance to proteolysis were selected as LEADs. The candidate LEADs that possessed at least as much residual antiviral activity following protease treatment as the control, native IFN $\alpha$ -2b, before protease treatment were selected as LEADs. The results are set forth in Table 2 of Example 2.

Using this method, the following mutants selected as LEADs are provided herein and correspond to the group of proteins containing one or more single amino acid replacements in SEQ ID NO:1, corresponding to: F by V at position 27; R by H at position 33; E by Q at position 41; E by H at position 41; E by Q at position 58; E by H at position 58; E by Q at position 78; E by H at position 78; Y by H at position 89; E by Q at position 107; E by H at position 107; P by A at position 109; L by V at position 110; M by V at position 111; E by Q at position 113; E by H at position 113; L by V at position 117; L by I at position 117; K by Q at position 121; K by T at position 121; R by H at position 125; R by Q at



-68-

position 125; K by Q at position 133; K by T at position 133; and E by Q at position 159; E by H at position 159. Among these are mutations that can have multiple effects. For example, among mutations described herein, are mutations that result in an increase of the IFN $\alpha$ -2b activity as  
5 assessed by detecting the requisite biological activity.

Also provided are IFN $\alpha$ -2b proteins that contain a plurality of mutations based on the LEADs (see, *e.g.*, Tables 6 and 7, EXAMPLE 5, which lists candidate LEADs and LEAD sites), are generated. These IFN $\alpha$ -2b proteins have activity that is further optimized. Examples of such  
10 proteins are described in the EXAMPLES. Other combinations of mutations can be prepared and tested as described herein to identify other LEADs of interest, particularly those that have further increased IFN $\alpha$ -2b antiviral activity or further increased resistance to proteolysis.

Also provided herein are modified IFN $\alpha$ -2b or IFN $\alpha$ -2a cytokines  
15 selected from the group consisting of proteins comprising one or more single amino acid replacements in SEQ ID NOS:1 or 182, corresponding to the replacement of: N by D at position 45 (*e.g.*, SEQ ID NO:978); D by G at position 94 (*e.g.*, SEQ ID NO:979); G by R at position 102 (*e.g.*, SEQ ID NO:980); A by G at position 139 (*e.g.*, SEQ ID NO:981); or any  
20 combination thereof. These particular proteins have also been found herein to have increased resistance to proteolysis.

In another embodiment, IFN $\alpha$ -2b and IFN $\alpha$ -2a proteins that contain a plurality of mutations based on the LEADs (see Tables in the EXAMPLES, listing the candidate LEADs and LEAD sites), are produced to  
25 produce IFN $\alpha$ -2b and IFN $\alpha$ -2a proteins that have activity that is further optimized. Examples of such proteins are described herein. Other combinations of mutations can be prepared and tested as described herein to identify other LEADs of interest, particularly those that have further

-69-

increased IFN $\alpha$ -2b and IFN $\alpha$ -2a antiviral activity or further increased resistance to proteolysis.

### 3) N-glycosylation Site Addition

In additional embodiments, N-glycosylation sites can be added to

- 5 increase resistance to proteolysis while maintaining or improving the requisite biological activity. Exemplary N-glycosylation mutants containing duo-amino acid replacements corresponding to the N-X-S or N-X-T consensus sequences are set forth in Example 3. Accordingly, provided herein are IFN $\alpha$ -2b and IFN $\alpha$ -2a mutant proteins having an
- 10 increased resistance to proteolysis compared to unmodified IFN $\alpha$ -2b and IFN $\alpha$ -2a, selected from the group consisting of proteins comprising one or more sets of duo-amino acid replacements in SEQ ID NO:1, corresponding to:
- D by N at position 2 and P by S at position 4;
- 15 D by N at position 2 and P by T at position 4;
- L by N at position 3 and Q by S at position 5;
- L by N at position 3 and Q by T at position 5;
- P by N at position 4 and T by S at position 6;
- P by N at position 4 and T by T at position 6;
- 20 Q by N at position 5 and H by S at position 7;
- Q by N at position 5 and H by T at position 7;
- T by N at position 6 and S by S at position 8;
- T by N at position 6 and S by T at position 8;
- H by N at position 7 and L by S at position 9;
- 25 H by N at position 7 and L by T at position 9;
- S by N at position 8 and G by S at position 10;
- S by N at position 8 and G by T at position 10;
- L by N at position 9 and S by S at position 11;
- L by N at position 9 and S by T at position 11;

-70-

- M by N at position 21 and K by S at position 23;  
M by N at position 21 and K by T at position 23;  
R by N at position 22 and I by S at position 24;  
R by N at position 22 and I by T at position 24;
- 5 K or R by N at position 23 and S by S at position 25;  
K or R by N at position 23 and S by T at position 25;  
I by N at position 24 and L by S at position 26;  
I by N at position 24 and L by T at position 26;  
S by N at position 25 and F by S at position 27;
- 10 S by N at position 25 and F by T at position 27;  
L by N at position 26 and S by S at position 28;  
L by N at position 26 and S by T at position 28;  
S by N at position 28 and L by S at position 30;  
S by N at position 28 and L by T at position 30;
- 15 L by N at position 30 and D by S at position 32;  
L by N at position 30 and D by T at position 32;  
K by N at position 31 and R by S at position 33;  
K by N at position 31 and R by T at position 33;  
D by N at position 32 and H by S at position 34;
- 20 D by N at position 32 and H by T at position 34;  
R by N at position 33 and D by S at position 35;  
R by N at position 33 and D by T at position 35;  
H by N at position 34 and F by S at position 36;  
H by N at position 34 and F by T at position 36;
- 25 D by N at position 35 and G by S at position 37;  
D by N at position 35 and G by T at position 37;  
F by N at position 36 and F by S at position 38;  
F by N at position 36 and F by T at position 38;  
G by N at position 37 and P by S at position 39;

-71-

- G by N at position 37 and P by T at position 39;  
F by N at position 38 and Q by S at position 40;  
F by N at position 38 and Q by T at position 40;  
P by N at position 39 and E by S at position 41;  
5 P by N at position 39 and E by T at position 41;  
Q by N at position 40 and E by S at position 42;  
Q by N at position 40 and E by T at position 42;  
E by N at position 41 and F by S at position 43;  
E by N at position 41 and F by T at position 43;  
10 E by N at position 42 and G by S at position 44;  
E by N at position 42 and G by T at position 44;  
F by N at position 43 and N by S at position 45;  
F by N at position 43 and N by T at position 45;  
G by N at position 44 and Q by S at position 46;  
15 G by N at position 44 and Q by T at position 46;  
N by N at position 45 and F by S at position 47;  
N by N at position 45 and F by T at position 47;  
Q by N at position 46 and Q by S at position 48;  
Q by N at position 46 and Q by T at position 48;  
20 F by N at position 47 and K by S at position 49;  
F by N at position 47 and K by T at position 49;  
Q by N at position 48 and A by S at position 50;  
Q by N at position 48 and A by T at position 50;  
K by N at position 49 and E by S at position 51;  
25 K by N at position 49 and E by T at position 51;  
A by N at position 50 and T by S at position 52;  
A by N at position 50 and T by T at position 52;  
S by N at position 68 and K by S at position 70;  
S by N at position 68 and K by T at position 70;

-72-

- K by N at position 70 and S by S at position 72;  
K by N at position 70 and S by T at position 72;  
A by N at position 75 and D by S at position 77;  
A by N at position 75 and D by T at position 77;  
5 D by N at position 77 and T by S at position 79;  
D by N at position 77 and T by T at position 79;  
I by N at position 100 and G by S at position 102;  
I by N at position 100 and G by T at position 102;  
Q by N at position 101 and V by S at position 103;  
10 Q by N at position 101 and V by T at position 103;  
G by N at position 102 and G by S at position 104;  
G by N at position 102 and G by T at position 104;  
V by N at position 103 and V by S at position 105;  
V by N at position 103 and V by T at position 105;  
15 G by N at position 104 and T by S at position 106;  
G by N at position 104 and T by T at position 106;  
V by N at position 105 and E by S at position 107;  
V by N at position 105 and E by T at position 107;  
T by N at position 106 and T by S at position 108;  
20 T by N at position 106 and T by T at position 108;  
E by N at position 107 and P by S at position 109;  
E by N at position 107 and P by T at position 109;  
T by N at position 108 and I by S at position 110;  
T by N at position 108 and I by T at position 110;  
25 K by N at position 134 and S by S at position 136;  
K by N at position 134 and S by T at position 136;  
S by N at position 154 and N by S at position 156;  
S by N at position 154 and N by T at position 156;  
T by N at position 155 and L by S at position 157;

-73-

- T by N at position 155 and L by T at position 157;  
N by N at position 156 and Q by S at position 158;  
N by N at position 156 and Q by T at position 158;  
L by N at position 157 and E by S at position 159;  
5 L by N at position 157 and E by T at position 159;  
Q by N at position 158 and S by S at position 160;  
Q by N at position 158 and S by T at position 160;  
E by N at position 159 and L by S at position 161;  
E by N at position 159 and L by T at position 161;  
10 S by N at position 160 and R by S at position 162;  
S by N at position 160 and R by T at position 162;  
L by N at position 161 and S by S at position 163;  
L by N at position 161 and S by T at position 163;  
R by N at position 162 and K by S at position 164;  
15 R by N at position 162 and K by T at position 164;  
S by N at position 163 and E by S at position 165; and  
S by N at position 163 and E by T at position 165,

wherein residue 1 corresponds to residue 1 of the mature IFN $\alpha$ -2b or IFN $\alpha$ -2a protein set forth in SEQ ID NO:1 or SEQ ID NO:182,

- 20 respectively. In particular embodiments, the IFN $\alpha$ -2b or IFN $\alpha$ -2a mutant protein has increased resistance to proteolysis compared to unmodified IFN $\alpha$ -2b or IFN $\alpha$ -2a, and is selected from the group consisting of proteins comprising one or more sets of duo-amino acid replacements in SEQ ID NO:1, corresponding to:  
25 Q by N at position 5 and H by S at position 7;  
P by N at position 39 and E by S at position 41;  
P by N at position 39 and E by T at position 41;  
Q by N at position 40 and E by S at position 42;  
Q by N at position 40 and E by T at position 42;

-74-

- E by N at position 41 and F by S at position 43;  
E by N at position 41 and F by T at position 43;  
F by N at position 43 and N by S at position 45;  
G by N at position 44 and Q by T at position 46;  
5 N by N at position 45 and F by S at position 47;  
N by N at position 45 and F by T at position 47;  
Q by N at position 46 and Q by S at position 48;  
F by N at position 47 and K by S at position 49;  
F by N at position 47 and K by T at position 49;  
10 I by N at position 100 and G by S at position 102;  
I by N at position 100 and G by T at position 102;  
V by N at position 105 and E by S at position 107;  
V by N at position 105 and E by T at position 107;  
T by N at position 106 and T by S at position 108;  
15 T by N at position 106 and T by T at position 108;  
E by N at position 107 and P by S at position 109;  
E by N at position 107 and P by T at position 109;  
L by N at position 157 and E by S at position 159;  
L by N at position 157 and E by T at position 159;  
20 E by N at position 159 and L by S at position 161; and  
E by N at position 159 and L by T at position 161.

**F. Protein Redesign**

Provided herein are methods for designing and generating new versions of native or modified cytokines, such as IFN $\alpha$ -2b and IFN $\alpha$ -2a.

- 25 Using these methods, the redesigned cytokine maintains either sufficient, typically equal or improved levels of a selected phenotype, such as a biological activity, of the original protein, while at the same time its amino acid sequence is changed by replacement of up to: at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at

-75-

least 8%, at least 9%, at least 10%, at least 12%, at least 14%, at least 16%, at least 18%, at least 20%, at least 30%, at least 40% up to 50% or more of its native amino acids by the appropriate pseudo-wild type amino acids. Pseudo-wild type amino acids are those amino acids such

5 that when they replace an original, such as native, amino acid at a given position on the protein sequence, the resulting protein displays substantially the same levels of biological activity (or sufficient activity for its therapeutic or other use) compared to the original, such as native, protein. In other embodiments, pseudo-wild type amino acids are those

10 amino acids such that when they replace an original, such as native, amino acid at a given position on the protein sequence, the resulting protein displays the same phenotype, such as levels of biological activity, compared to an original, typically a native, protein. Pseudo-wild type amino acids and the appropriate replacing positions can be detected and

15 identified by any analytical or predictive means; such as for example, by performing an Alanine-scanning. Any other amino acid, particularly another amino acid that has a neutral effect on structure, such as Gly or Ser, also can be used for the scan. All those replacements of original, such as native, amino acids by Ala that do not lead to the generation of a

20 HIT (a protein that has lost the desired biological activity), have either led to the generation of a LEAD (a protein with increased biological activity); or the replacement by Ala will be a neutral replacement, i.e., the resulting protein will display comparable levels of biological activity compared to the original, such as native, protein. The methods provided herein for

25 protein redesign of cytokines, such as IFN $\alpha$ -2b and IFN $\alpha$ -2a, are intended to design and generate "artificial" (versus naturally existing) proteins, such that they consist of amino acid sequences not existing in Nature, but that display biological activities characteristic of the original, such as native, protein. These redesigned proteins are contemplated herein to be



-76-

useful for avoiding potential side effects that might otherwise exist in other forms of cytokines in treatment of disease. Other uses of redesigned proteins provided herein are to establish cross-talk between pathways triggered by different proteins; to facilitate structural biology by  
5 generating mutants that can be crystallized while maintaining activity; and to destroy an activity of a protein without changing a second activity or multiple additional activities.

In one embodiment, a method for obtaining redesigned proteins comprises *i*) identifying some or all possible target sites on the protein  
10 sequence that are susceptible to amino acid replacement without losing protein activity (protein activity in a largest sense of the term: enzymatic, binding, hormone, etc.) (These sites are the pseudo-wild type,  $\Psi$ -wt sites); *ii*) identifying appropriate replacing amino acids ( $\Psi$ -wt amino acids), specific for each  $\Psi$ -wt site, such that if used to replace the native amino  
15 acids at that specific  $\Psi$ -wt site, they can be expected to generate a protein with comparable biological activity compared to the original, such as native, protein, thus keeping the biological activity of the protein substantially unchanged; *iii*) systematically introducing the specific  $\Psi$ -wt amino acids at every specific  $\Psi$ -wt position so as to generate a collection  
20 containing the corresponding mutant molecules. Mutants are generated, produced and phenotypically characterized one-by-one, in addressable arrays, such that each mutant molecule contains initially amino acid replacements at only one  $\Psi$ -wt site. In subsequent rounds mutant molecules also can be generated such that they contain one or more  $\Psi$ -wt  
25 amino acids at one or more  $\Psi$ -wt sites. Those mutant proteins carrying several mutations at a number of  $\Psi$ -wt sites, and that display comparable or improved biological activity are called redesigned proteins or  $\Psi$ -wt proteins. In particular embodiments, at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at

-77-

least 9%, at least 10%, at least 15%, at least 20%, at least 25%, or more of the amino acid residue positions on a particular cytokine, such as IFN $\alpha$ -2b and IFN $\alpha$ -2a are replaced with an appropriate pseudo-wild type amino acid.

- 5           The first step is an amino acid scan over the full length of the protein. At this step, each and every one of the amino acids in the protein sequence is replaced by a selected reference amino acid, such as alanine. This permits the identification of "redesign-HIT" positions, i.e., positions that are sensitive to amino acid replacement. All of the other
- 10 positions that are not redesign-HIT positions (i.e., those at which the replacement of the original, such as native, amino acid by the replacing amino acid, for example Ala, does not lead to a drop in protein fitness or biological activity) are referred to herein as "pseudo-wild type" positions. When the replacing amino acid, for example Ala, replaces the original,
- 15 such as native, amino acid at a non-HIT position, then the replacement is neutral, in terms of protein activity, and the replacing amino acid is said to be a pseudo-wild type amino acid at that position. Pseudo-wild type positions appear to be less sensitive than redesign-HIT positions since they tolerate the amino acid replacement without affecting the protein
- 20 activity that is being either maintained or improved. Amino acid replacement at the pseudo-wild type positions, result in a non-change in the protein fitness (e.g., possess substantially the same biological activity), while at the same time to a divergence in the resulting protein sequence compared to the original, such as native, sequence.
- 25           To first identify those amino acid positions on the IFN $\alpha$ -2b and IFN $\alpha$ -2a protein that are involved or not involved in IFN $\alpha$ -2b and IFN $\alpha$ -2a protein activity, such as binding activity of IFN $\alpha$ -2b and IFN $\alpha$ -2a to its receptor, an Ala-scan was performed on the IFN $\alpha$ -2b sequence as set forth in Example 4. For this purpose, each amino acid in the IFN $\alpha$ -2b

-78-

protein sequence was individually changed to Alanine. Any other amino acid, particularly another amino acid that has a neutral effect on structure, such as Gly or Ser, also can be used. Each resulting mutant IFN $\alpha$ -2b protein was then expressed and the activity of the interferon

5 molecule was then assayed. These particular amino acid positions, referred to herein as HITs would in principle not be suitable targets for amino acid replacement to increase protein stability, because of their involvement in the recognition of IFN-receptor or in the downstream pathways involved in IFN activity. For the Ala-scanning, the biological

10 activity measured for the IFN $\alpha$ -2b molecules was: *i*) their capacity to inhibit virus replication when added to permissive cells previously infected with the appropriate virus and, *ii*) their capacity to stimulate cell proliferation when added to the appropriate cells. The relative activity of each individual mutant compared to the native protein is indicated in

15 FIG10A through C. HITs are those mutants that produce a decrease in the activity of the protein (in the example: all the mutants with activities below about 30% of the native activity).

In addition, the Alanine-scan was used to identify the amino acid residues on IFN $\alpha$ -2b that when replaced with alanine correspond to

20 'pseudo-wild type' activity, i.e., those that can be replaced by alanine without leading to a decrease in biological activity. Knowledge of these amino acids is useful for the re-design of the IFN $\alpha$ -2b and IFN $\alpha$ -2a proteins. The results are set forth in Table 5, and include pseudo-wild type amino acid positions of IFN $\alpha$ -2b corresponding to SEQ ID NO:1,

25 amino acid residues: 9, 10, 17, 20, 24, 25, 35, 37, 41, 52, 54, 56, 57, 58, 60, 63, 64, 65, 76, 89, and 90.

Accordingly, provided herein are IFN $\alpha$ -2b and IFN $\alpha$ -2a mutant proteins comprising one or more pseudo-wild type mutations at amino acid positions of IFN $\alpha$ -2b or IFN $\alpha$ -2a corresponding to SEQ ID NO:1 or

-79-

SEQ ID NO:182, respectively, amino acid residues: 9, 10, 17, 20, 24, 25, 35, 37, 41, 52, 54, 56, 57, 58, 60, 63, 64, 65, 76, 89, and 90. The mutations can be either one or more of insertions, deletions and/or replacements of the native amino acid residue(s). In one embodiment, the  
5 pseudo-wild type replacements are mutations with alanine at each position. In another embodiment, the pseudo-wild type replacements are one or more mutations in SEQ ID NO:1 corresponding to:

L by A at position 9, L by A at position 17,  
Q by A at position 20, I by A at position 24,  
10 S by A at position 25, D by A at position 35,  
G by A at position 37, E by A at position 41,  
T by A at position 52, P by A at position 54,  
L by A at position 56, H by A at position 57,  
E by A at position 58, I by A at position 60,  
15 I by A at position 63, F by A at position 64,  
N by A at position 65, W by A at position 76,  
Y by A at position 89, and Q by A at position 90.

In addition, the IFN $\alpha$ -2b alanine scan revealed the following redesign-HITs having decreased antiviral activity at amino acid positions  
20 of IFN $\alpha$ -2b corresponding to SEQ ID NO:1, amino acid residues: 2, 7, 8, 11, 13, 15, 16, 23, 26, 28, 29, 30, 31, 32, 33, 53, 69, 91, 93, 98, and 101. Accordingly, in particular embodiments where it is desired to decrease the viral activity of IFN $\alpha$ -2b or IFN $\alpha$ -2a, either one or more of insertions, deletions and/or replacements of the native amino acid  
25 residue(s) can be carried out at one or more of amino acid positions of IFN $\alpha$ -2b or IFN $\alpha$ -2a corresponding to SEQ ID NO:1, amino acid residues: 2, 7, 8, 11, 13, 15, 16, 23, 26, 28, 29, 30, 31, 32, 33, 53, 69, 91, 93, 98, and 101.

-80-

Each of the redesign mutations set forth above can be combined with one or more of the IFN $\alpha$ -2b or IFN $\alpha$ -2a candidate LEAD mutations or one or more of the IFN $\alpha$ -2b or IFN $\alpha$ -2a LEAD mutants provided herein.

**G. 3D-scanning and Its Use for Modifying Cytokines**

5 Also provided herein is a method of structural homology analysis for comparing proteins regardless of their underlying amino acid sequences. For a subset of proteins families, such as the family of human cytokines, this information is rationally exploited to produce modified proteins. This method of structural homology analysis can be  
10 applied to proteins that are evolved by any method, including the 2D scanning method described herein. When used with the 2D method in which a particular phenotype, activity or characteristic of a protein is modified by 2D analysis, the method is referred to as 3D-scanning.

The use of "structural homology" analysis in combination with the  
15 directed evolution methods provided herein provides a powerful technique for identifying and producing various new protein mutants, such as cytokines, having desired biological activities, such as increased resistance to proteolysis. For example, the analysis of the "structural homology" between an optimized mutant version of a given protein and  
20 "structurally homologous" proteins allows identification of the corresponding structurally related or structurally similar amino acid positions (also referred to herein as "structurally homologous loci") on other proteins. This permits identification of mutant versions of the latter that have a desired optimized feature(s) (biological activity, phenotype) in  
25 a simple, rapid and predictive manner (regardless of amino acid sequence and sequence homology). Once a mutant version of a protein is developed, then, by applying the rules of structural homology, the corresponding structurally related amino acid positions (and replacing amino acids) on other "structurally homologous" proteins readily are

-81-

identified, thus allowing a rapid and predictive discovery of the appropriate mutant versions for the new proteins.

3-dimensionally structurally equivalent or similar amino acid positions that are located on two or more different protein sequences that

5 share a certain degree of structural homology, have comparable functional tasks (activities and phenotypes). These two amino acids that occupy substantially equivalent 3-dimensional structural space within their respective proteins can then be said to be "structurally similar" or "structurally related" with each other, even if their precise positions on

10 the amino acid sequences, when these sequences are aligned, do not match with each other. The two amino acids also are said to occupy "structurally homologous loci." "Structural homology" does not take into account the underlying amino acid sequence and solely compares 3-dimensional structures of proteins. Thus, two proteins can be said to

15 have some degree of structural homology whenever they share conformational regions or domains showing comparable structures or shapes with 3-dimensional overlapping in space. Two proteins can be said to have a higher degree of structural homology whenever they share a higher amount of conformational regions or domains showing

20 comparable structures or shapes with 3-dimensional overlapping in space. Amino acids positions on one or more proteins that are "structurally homologous" can be relatively far way from each other in the protein sequences, when these sequences are aligned following the rules of primary sequence homology. Thus, when two or more protein backbones

25 are determined to be structurally homologous, the amino acid residues that are coincident upon three-dimensional structural superposition are referred to as "structurally similar" or "structurally related" amino acid residues in structurally homologous proteins (also referred to as "structurally homologous loci"). Structurally similar amino acid residues

-82-

are located in substantially equivalent spatial positions in structurally homologous proteins.

For example, for proteins of average size (approximately 180 residues), two structures with a similar fold will usually display rms  
5 deviations not exceeding 3 to 4 angstroms. For example, structurally similar or structurally related amino acid residues can have backbone positions less than 3.5, 3.0, 2.5, 2.0, 1.7 or 1.5 angstrom from each other upon protein superposition. RMS deviation calculations and protein superposition can be carried out using any of a number of methods  
10 known in the art. For example, protein superposition and RMS deviation calculations can be carried out using all peptide backbone atoms (e.g., N, C, C(C=O), O and CA (when present)). Alternatively, protein superposition can be carried out using just one or any combination of peptide backbone atoms, such as, for example, N, C, C(C=O), O and CA  
15 (when present). In addition, one skilled in the art will recognize that protein superposition and RMS deviation calculations generally can be performed on only a subset of the entire protein structure. For example, if the protein superposition is carried out using one protein that has many more amino acid residues than another protein, protein superposition can  
20 be carried out on the subset (e.g., a domain) of the larger protein that adopts a structure similar to the smaller protein. Similarly, only portions of other proteins can be suitable for superimposition. For example, if the position of the C-terminal residues from two structurally homologous proteins differ significantly, the C-terminal residues can be omitted from  
25 the structural superposition or RMS deviation calculations.

Accordingly, provided herein are methods of rational evolution of proteins based on the identification of potential target sites for mutagenesis (is-HITs) through comparison of patterns of protein backbone folding between structurally related proteins, irrespective of the

-83-

underlying sequences of the compared proteins. Once the structurally related amino acid positions are identified on the new protein, then suitable amino acid replacement criteria, such as PAM analysis, can be employed to identify candidate LEADs for construction and screening as  
5 described herein.

For example, analysis of "structural homology" between and among a number of related cytokines was used to identify on various members of the cytokine family, other than interferon alpha, those amino acid positions and residues that are structurally similar or structurally  
10 related to those found in the IFN $\alpha$ -2b mutants provided herein that have been optimized for improved stability. The resulting modified cytokines are provided. This method can be applied to any desired phenotype using any protein, such as a cytokine, as the starting material to which an evolution procedure, such as the rational directed evolution procedure of  
15 U.S. application Serial No. 10/022,249 or the 2-dimensional scanning method provided herein, is applied. The structurally corresponding residues are then altered on members of the family to produce additional cytokines with similar phenotypic alterations.

#### 1) Homology

20 Typically, homology between proteins is compared at the level of their amino acid sequences, based on the percent or level of coincidence of individual amino acids, amino acid per amino acid, when sequences are aligned starting from a reference, generally the residue encoded by the start codon. For example, two proteins are said to be "homologous" or to  
25 bear some degree of homology whenever their respective amino acid sequences show a certain degree of matching upon alignment comparison. Comparative molecular biology is primarily based on this approach. From the degree of homology or coincidence between amino acid sequences, conclusions can be made on the evolutionary distance



-84-

between or among two or more protein sequences and biological systems.

The concept of "convergent evolution" is applied to describe the phenomena by which phylogenetically unrelated organisms or biological  
5 systems have evolved to share features related to their anatomy, physiology and structure as a response to common forces, constraints, and evolutionary demands from the surrounding environment and living organisms. Alternatively, "divergent evolution," is applied to describe the phenomena by which strongly phylogenetically related organisms or  
10 biological systems have evolved to diverge from identity or similarity as a response to divergent forces, constraints, and evolutionary demands from the surrounding environment and living organisms.

In the typical traditional analysis of homologous proteins there are two conceptual biases corresponding to: i) "convergent evolution," and  
15 ii) "divergent evolution." Whenever the aligned amino acid sequences of two proteins do not match well with each other, these proteins are considered "not related" or "less related" with each other and have different phylogenetic origins. There is no (or low) homology between these proteins and their respective genes are not homologous (or show  
20 little homology). If these two "non-homologous" proteins under study share some common functional features (e.g., interaction with other specific molecules, activity), they are determined to have arisen by "convergent evolution," i.e., by evolution of their non-homologous amino acid sequences, in such a way that they end up generating functionally  
25 "related" structures.

On the other hand, whenever the aligned amino acid sequences of two proteins do match with each other to a certain degree, these proteins are considered to be "related" and to share a common phylogenetic origin. A given degree of homology is assigned between these two

-85-

proteins and their respective genes likewise share a corresponding degree of homology. During the evolution of their initial highly homologous amino acid sequence, enough changes can be accumulated in such a way that they end up generating "less-related" sequences and less related  
5 function. The divergence from perfect matching between these two "homologous" proteins under study is said come from "divergent evolution."

**2) 3D-scanning (Structural Homology) methods**

Structural homology refers to homology between the  
10 topology and three-dimensional structure of two proteins. Structural homology is not necessarily related to "convergent evolution" or to "divergent evolution," nor is it related to the underlying amino acid sequence. Rather, structural homology is likely driven (through natural evolution) by the need of a protein to fit specific conformational demands  
15 imposed by its environment. Particular structurally homologous "spots" or "loci" would not be allowed to structurally diverge from the original structure, even when its own underlying sequence does diverge. This structural homology is exploited herein to identify loci for mutation.

Within the amino acid sequence of a protein resides the appropriate  
20 biochemical and structural signals to achieve a specific spatial folding in either an independent or a chaperon-assisted manner. Indeed, this specific spatial folding ultimately determines protein traits and activity. Proteins interact with other proteins and molecules in general through their specific topologies and spatial conformations. In principle, these  
25 interactions are not based solely on the precise amino acid sequence underlying the involved topology or conformation. If protein traits, activity (behavior and phenotypes) and interactions rely on protein topology and conformation, then evolutionary forces and constraints acting on proteins can be expected to act on topology and conformation.

-86-

Proteins sharing similar functions will share comparable characteristics in their topology and conformation, despite the underlying amino acid sequences that create those topologies and conformations.

### 3) Application of 3D Scanning to Cytokines

5       The method based on structural homology, including the 3D-scanning method provided herein can be applied to any related proteins. For exemplary purposes herein it is applied to cytokines. In exemplary embodiments, methods for altering phenotypes of members of families of cytokines by altering one member such as by employing the 2-  
10       dimensional rational scanning method are provided. As provided herein, other members of these cytokine families then can be similarly modified by identifying and changing structurally homologous residues to similarly alter the phenotypes of such proteins.

      In an exemplary embodiment herein, IFN $\alpha$ -2b mutants with  
15       increased resistance to proteolysis are generated by the 2-dimensional rational scanning method; IFN $\beta$  mutants also were generated. The corresponding residues on members of cytokine families that possess structural homology to IFN $\alpha$ -2b were identified and the identified residues on the other cytokines were similarly modified to produce cytokines with  
20       increased resistance to proteolysis. Hence also provided herein are cytokine mutants that display increased resistance to proteolysis and/or glomerular filtration containing one or more amino acid replacements.

      Provided herein are mutant (modified) cytokines that display altered features and properties, such as a resistance to proteolysis. Methods for  
25       producing such modified cytokines also are provided.

      Also provided herein is a method of structural homology analysis for comparing proteins regardless their underlying amino acid sequences. For a subset of proteins families, such as the family of human cytokines, this information is rationally exploited herein. Human cytokines all share a

-87-

common helix bundle fold, which is used to structurally define the 4-helical cytokine superfamily in the structural classification of the protein database SCOP® (Structural Classification of Proteins; see, e.g., Murzin *et al.*, *J. Mol. Biol.*, 247:536-540, 1995 and "[http://scop.mrc-](http://scop.mrc-lmb.cam.ac.uk/scop/)

- 5 [lmb.cam.ac.uk/scop/](http://scop.mrc-lmb.cam.ac.uk/scop/)"). This superfamily includes three different families: 1) the interferons/interleukin-10 protein family (SEQ ID NOS: 1 and 182-200); 2) the long-chain cytokine family (SEQ ID NOS: 210-217); and 3) the short-chain cytokine family (SEQ ID NOS: 201-209).

- For example, a distinct feature of cytokines from the inter-
- 10 ferons/interleukin-10 family is an additional (fifth) helix. This family includes interleukin-10 (IL-10; SEQ ID NO:200, interferon beta (IFN $\beta$ ; SEQ ID NO: 196), interferon alpha-2a (IFN $\alpha$ -2a; SEQ ID NO: 182), interferon alpha-2b (IFN $\alpha$ -2b; SEQ ID NO:1), and interferon gamma (IFN- $\gamma$ ; SEQ ID NO: 199). The long-chain cytokine protein family includes, among others,
- 15 granulocyte colony stimulating factor (G-CSF; SEQ ID NO: 210), leukemia inhibitory factor (LIF; SEQ ID NO: 213), growth hormone (hGH; SEQ ID NO: 216), ciliary neurotrophic factor (CNTF; SEQ ID NO: 212), leptin (SEQ ID NO: 211), oncostatin M (SEQ ID NO: 214), interleukin-6 (IL-6; SEQ ID NO: 217) and interleukin-12 (IL-12; SEQ ID NO: 215). The short-
- 20 chain cytokine protein family includes, among others, erythropoietin (EPO; SEQ ID NO: 201), granulocyte-macrophage colony stimulating factor (GM-CSF; SEQ ID NO: 202), interleukin-2 (IL-2; SEQ ID NO: 204), interleukin-3 (IL-3; SEQ ID NO: 205), interleukin-4 (IL-4; SEQ ID NO: 207), interleukin-5 (IL-5; SEQ ID NO: 208), interleukin-13 (IL-13; SEQ ID NO: 209), Flt3
- 25 ligand (SEQ ID NO: 203) and stem cell factor (SCF; SEQ ID NO: 206).

Although the degree of similarity among the underlying amino acid sequences of these cytokines does not appear high, their corresponding 3-dimensional structures present a high level of similarity (see, e.g., FIGS8B through D). Effectively, the best structural similarity is obtained

-88-

between two 3-dimensional protein structures of the same family in the 4-helical cytokine superfamily.

The methods provided herein for producing mutant cytokines are exemplified with reference to production of cytokines that display a  
5 substantially equivalent increase in resistance to proteolysis relative to the optimized IFN $\alpha$ -2b mutants. It is understood that this method can be applied to other families of proteins and for other phenotypes.

In one embodiment, proteins of the 4-helical cytokine superfamily are provided herein that are structurally homologous IFN $\alpha$ -2b LEAD  
10 mutants set forth herein. For example, by virtue of the knowledge of the 3-dimensional structural amino acid positions within the LEAD IFN $\alpha$ -2b mutants provided herein that confer higher resistance to a challenge with either proteases or blood lysate or serum, while maintaining or improving the requisite biological activity, the corresponding structurally related  
15 (e.g., structurally similar) amino acid residues on a variety of other cytokines are identified (FIG9).

Numerous methods are well known in the art for identifying structurally related amino acid positions with 3-dimensionally structurally homologous proteins. Exemplary methods include, but are not limited to:  
20 CATH (Class, Architecture, Topology and Homologous superfamily) which is a hierarchical classification of protein domain structures based on four different levels (Orengo *et al.*, *Structure*, 5(8):1093-1108, 1997); CE (Combinatorial Extension of the optimal path), which is a method that calculates pairwise structure alignments (Shindyalov *et al.*, *Protein*  
25 *Engineering*, 11(9):739-747, 1998); FSSP (Fold classification based on Structure-Structure alignment of Proteins), which is a database based on the complete comparison of all 3-dimensional protein structures that currently reside in the Protein Data Bank (PDB) (Holm *et al.*, *Science*, 273:595-602, 1996); SCOP (Structural Classification of Proteins), which

-89-

provides a descriptive database based on the structural and evolutionary relationships between all proteins whose structure is known (Murzin *et al.*, *J. Mol. Biol.*, 247:536-540, 1995); and VAST (Vector Alignment Search Tool), which compares newly determined 3-dimensional protein  
5 structure coordinates to those found in the MMDB/PDB database (Gibrat *et al.*, *Current Opinion in Structural Biology*, 6:377-385, 1995).

In a particular embodiment, the step-by-step process including the use of a program referred to as TOP (see FIG8A and Lu, G., *J. Appl. Cryst.*, 33:176-189, 2000; publicly available at  
10 "[www.bioinfo1.mbfys.lu.se/TOP](http://www.bioinfo1.mbfys.lu.se/TOP)") is used for protein structure comparison. This program runs two steps for each protein structure comparison. In the first step topology of secondary structure in the two structures is compared. The program uses two points to represent each secondary structure element (alpha helices or beta strands) then  
15 systematically searches all the possible super-positions of these elements in 3-dimensional space (defined as the root mean square deviation – rmsd, the angle between the two lines formed by the two points and the line-line distance). The program searches to determine whether additional secondary structure elements can fit by the same superposition operation.  
20 If secondary structures that can fit each other exceed a given number, the program identifies the two structures as similar. The program gives as an output a comparison score called "Structural Diversity" that considers the distance between matched  $\alpha$ -carbon atoms and the number of matched residues. The lower the "Structural Diversity" score, the more the two  
25 structures are similar. In various embodiments herein, the Structural Diversity scores range from 0 up to about 67.

In the exemplified embodiment, all the cytokines were first structurally aligned against the IFN $\alpha$ -2b structure. For the proteins within

-90-

the same family as IFN $\alpha$ -2b (e.g., the interferons/interleukin-10 cytokine family), this alignment was directly used to identify the structurally related is-HIT target amino acid positions and/or regions corresponding to the structurally homologous positions and/or regions on IFN $\alpha$ -2b where LEAD

5 mutants were found (FIG8B). For the other cytokines, the protein of the family (either long- or short-chain cytokines) with the best 3-dimensional structural alignment with IFN $\alpha$ -2b was selected using the lowest "Structural Diversity" score as the representative for that family. From the short-chain cytokine protein family, erythropoietin (EPO; see FIG8C)

10 was identified as the best structural homologue of IFN $\alpha$ -2b (rmsd = 1.9 angstroms; number of aligned residues = 62; Structural Diversity = 13.8). From the long-chain cytokine protein family, granulocyte-colony stimulating factor (G-CSF; see FIG8D) was identified as the best structural homologue of IFN $\alpha$ -2b (rmsd = 1.7 angstroms; number of aligned

15 residues = 77; Structural Diversity = 7.8).

Next, the amino acid positions and/or regions corresponding to the LEAD mutant regions on IFN $\alpha$ -2b were identified on these two proteins. These two best structural homologues of IFN $\alpha$ -2b (e.g., EPO and G-CSF; see FIGS12L and 12E, respectively) were structurally aligned to each of

20 the other cytokines within their respective cytokine protein families. As a result, protein regions likely to be targets for serum protease resistance were identified on all cytokines (see FIGS12A through T). Amino acids in these target regions were then checked for their exposure to the solvent and their susceptibility to be protease substrate. Exposed and substrate

25 residues are then subjected to PAM250 analysis as set forth above, so that a group of non-substrate and functionally conservative amino acid residues are selected as replacements. The results of the above structural homology analysis for each of the cytokines provided herein are set forth in FIGS12A through T.

-91-

Accordingly, provided herein are modified cytokines that exhibit greater resistance to proteolysis compared to the unmodified cytokine protein, comprising one or more amino acid replacements at one or more target positions on the cytokine corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of an IFN $\alpha$ -2b modified protein provided herein. The resistance to proteolysis can be measured by mixing it with a protease in vitro, incubation with blood or incubation with serum. Also provided herein are cytokine structural homologues of an IFN $\alpha$ -2b modified protein provided herein, comprising one or more amino acid replacements in the cytokine structural homologue at positions corresponding to the 3-dimensional-structurally-similar modified positions within the 3-dimensional structure of the modified IFN $\alpha$ -2b. In one embodiment, the cytokine homologue has increased resistance to proteolysis compared to its unmodified and/or wild type cytokine counterpart, wherein the resistance to proteolysis is measured by mixture with a protease in vitro, incubation with blood, or incubation with serum.

**a) Structurally Homologous Interferon Mutants**

Also provided herein are modified cytokines or cytokine structural homologues of IFN $\alpha$ -2b that are IFN $\alpha$  cytokines. These IFN $\alpha$  cytokines can be selected from the group consisting of IFN $\alpha$ -2a, IFN $\alpha$ -c, IFN $\alpha$ -2c, IFN $\alpha$ -d, IFN $\alpha$ -5, IFN $\alpha$ -6, IFN $\alpha$ -4, IFN $\alpha$ -4b, IFN $\alpha$ -l, IFN $\alpha$ -J, IFN $\alpha$ -H, IFN $\alpha$ -F, IFN $\alpha$ -8, and IFN $\alpha$ -consensus cytokine. Accordingly, the modified IFN $\alpha$  cytokines provided herein comprise one or more amino acid replacements at one or more target positions in either IFN $\alpha$ -2a, IFN $\alpha$ -c, IFN $\alpha$ -2c, IFN $\alpha$ -d, IFN $\alpha$ -5, IFN $\alpha$ -6, IFN $\alpha$ -4, IFN $\alpha$ -4b, IFN $\alpha$ -l, IFN $\alpha$ -J, IFN $\alpha$ -H, IFN $\alpha$ -F, IFN $\alpha$ -8, or IFN $\alpha$ -consensus cytokine corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of the IFN $\alpha$ -2b modified proteins provided herein, wherein the replacements lead



-92-

to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN alpha-2a.

- 5           In particular embodiments, the modified IFN $\alpha$  cytokines are selected from the group consisting of:
- the modified IFN $\alpha$ -2a that is human and is selected from the group consisting of proteins comprising one or more single amino acid replacements in SEQ ID NO: 182, corresponding to amino acid positions:
- 10   41, 58, 78, 107, 117, 125, 133 and 159;
- the modified IFN $\alpha$ -c that is human and is selected from the group consisting of proteins comprising one or more single amino acid replacements in SEQ ID NO: 183, corresponding to amino acid positions:
- 41, 59, 79, 108, 118, 126, 134 and 160;
- 15           the modified IFN $\alpha$ -2c cytokine that is human and is selected from the group consisting of cytokines comprising one or more single amino acid replacements in SEQ ID NO: 185, corresponding to amino acid positions: 41, 58, 78, 107, 117, 125, 133 and 159;
- the IFN $\alpha$ -d modified protein that is human and is selected from the
- 20   group consisting of proteins comprising one or more single amino acid replacements in SEQ ID NO: 186, corresponding to amino acid positions: 41, 59, 79, 108, 118, 126, 134 and 160;
- the IFN $\alpha$ -5 modified protein that is human and is selected from the group consisting of proteins comprising one or more single amino acid
- 25   replacements in SEQ ID NO: 187, corresponding to amino acid positions: 41, 59, 79, 108, 118, 126, 134 and 160;
- the IFN $\alpha$ -6 modified protein that is human and is selected from the group consisting of proteins comprising one or more single amino acid

-93-

replacements in SEQ ID NO: 188, corresponding to amino acid positions:  
41, 59, 79, 108, 118, 126, 134 and 160;

the IFN $\alpha$ -4 modified protein that is human and is selected from the  
group consisting of proteins comprising one or more single amino acid

5 replacements in SEQ ID NO: 189, corresponding to amino acid positions:  
41, 59, 79, 108, 118, 126, 134 and 160;

the IFN $\alpha$ -4b modified protein that is human and is selected from the  
group consisting of proteins comprising one or more single amino acid

10 replacements in SEQ ID NO: 190, corresponding to amino acid positions:  
41, 59, 79, 108, 118, 126, 134 and 160;

the IFN $\alpha$ -I modified protein that is human and is selected from the  
group consisting of proteins comprising one or more single amino acid

replacements in SEQ ID NO: 191, corresponding to amino acid positions:  
41, 59, 79, 108, 118, 126, 134 and 160;

15 the IFN $\alpha$ -J modified protein that is human and is selected from the  
group consisting of proteins comprising one or more single amino acid

replacements in SEQ ID NO: 192, corresponding to amino acid positions:  
41, 59, 79, 108, 118, 126, 134 and 160;

20 the IFN $\alpha$ -H modified protein that is human and is selected from the  
group consisting of proteins comprising one or more single amino acid

replacements in SEQ ID NO: 193, corresponding to amino acid positions:  
41, 59, 79, 108, 118, 126, 134 and 160;

the IFN $\alpha$ -F modified protein that is human and is selected from the  
group consisting of proteins comprising one or more single amino acid

25 replacements in SEQ ID NO: 194, corresponding to amino acid positions:  
41, 59, 79, 108, 118, 126, 134 and 160;

the IFN $\alpha$ -8 modified protein that is human and is selected from the  
group consisting of proteins comprising one or more single amino acid

-94-

replacements in SEQ ID NO: 195, corresponding to amino acid positions: 41, 59, 79, 108, 118, 126, 134 and 160; and

the IFN $\alpha$ -consensus modified protein that is human and is selected from the group consisting of proteins comprising one or more single  
5 amino acid replacements in SEQ ID NO: 232, corresponding to amino acid positions: 41, 58, 78, 107, 117, 125, 133 and 159.

**b) Structurally Homologous Cytokine Mutants**

As set forth above, provided herein are modified cytokines that comprise one or more amino acid replacements at one or more target  
10 positions in either interleukin-10 (IL-10), interferon beta (IFN $\beta$ ), IFN $\beta$ -1, IFN $\beta$ -2a, interferon gamma (IFN- $\gamma$ ), granulocyte colony stimulating factor (G-CSF), and human erythropoietin (EPO); corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of the IFN $\alpha$ -2b modified proteins provided herein, wherein the replacements  
15 lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified cytokine.

Also provided herein are modified cytokines that comprise one or more amino acid replacements at one or more target positions in either  
20 granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-13 (IL-13), Flt3 ligand and stem cell factor (SCF); corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of the human EPO modified proteins provided  
25 herein, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified cytokine.

Also provided herein are modified cytokines that comprise one or more amino acid replacements at one or more target positions in either

-95-

interleukin-10 (IL-10), interferon beta (IFN $\beta$ ), interferon gamma (IFN- $\gamma$ ), human granulocyte colony stimulating factor (G-CSF), leukemia inhibitory factor (LIF), human growth hormone (hGH), ciliary neurotrophic factor (CNTF), leptin, oncostatin M, interleukin-6 (IL-6) and interleukin-12 (IL-  
5 12); corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of the human G-CSF modified proteins provided herein, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified cytokine.

10 In particular embodiments, the modified cytokines are selected from the following.

A modified IFN $\beta$  cytokine, comprising mutations at one or more amino acid residues of IFN $\beta$  corresponding to SEQ ID NO: 196: 39, 42, 45, 47, 52, 67, 71, 73, 81, 107, 108, 109, 110, 111, 113, 116, 120,  
15 123, 124, 128, 130, 134, 136, 137, 163 and 165, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 196 set forth in FIG12A corresponding to SEQ ID NOS: 233-289,  
20 wherein the first amino acid indicated is substituted by the second at the position indicated for all of the substitutions set forth in FIG12A through T.

A modified IFN $\beta$ -1 cytokine, comprising mutations at one or more amino acid residues of IFN $\beta$ -1 corresponding to SEQ ID NO: 197: 39, 42,  
25 45, 47, 52, 67, 71, 73, 81, 107, 108, 109, 110, 111, 113, 116, 120, 123, 124, 128, 130, 134, 136, 137, 163 and 165, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

-96-

A modified IFN $\beta$ -2a cytokine, comprising mutations at one or more amino acid residues of IFN $\beta$ -2a corresponding to SEQ ID NO:198: 39, 42, 45, 47, 52, 67, 71, 73, 81, 107, 108, 109, 110, 111, 113, 116, 120, 123, 124, 128, 130, 134, 136, 137, 163 and 165, wherein the  
5 mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

A modified IFN-gamma cytokine, comprising mutations at one or more amino acid residues of IFN-gamma corresponding to SEQ ID NO:199: 33, 37, 40, 41, 42, 58, 61, 64, 65 and 66, wherein the  
10 mutations comprise insertions, deletions or replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO:199 set forth in FIG12B corresponding to SEQ ID NOS: 290-311.

A modified IL-10 cytokine, comprising mutations at one or more  
15 amino acid residues of IL-10 corresponding to SEQ ID NO:200: 49, 50, 52, 53, 54, 55, 56, 57, 59, 60, 67, 68, 71, 72, 74, 75, 78, 81, 84, 85, 86, and 88, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of  
20 amino acid substitutions in SEQ ID NO:200 set forth in FIG12C corresponding to SEQ ID NOS: 312-361.

A modified erythropoietin cytokine, comprising mutations at one or more amino acid residues of erythropoietin corresponding to SEQ ID NO:201: 43, 45, 48, 49, 52, 53, 55, 72, 75, 76, 123, 129, 130, 131,  
25 162, and 165, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 201 set forth in FIG12L corresponding to SEQ ID NOS: 940-977.

-97-

A modified GM-CSF cytokine, comprising mutations at one or more amino acid residues of GM-CSF corresponding to SEQ ID NO: 202: 38, 41, 45, 46, 48, 49, 51, 60, 63, 67, 92, 93, 119, 120, 123, and 124, wherein the mutations comprise insertions, deletions or replacements of  
5 the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 202 set forth in FIG12N corresponding to SEQ ID NOS: 362-400.

A modified Flt3 ligand cytokine, comprising mutations at one or  
10 more amino acid residues of Flt3 ligand corresponding to SEQ ID NO: 203: 3, 40, 42, 43, 55, 58, 59, 61, 89, 90, 91, 95, and 96, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID  
15 NO: 203 set forth in FIG12M corresponding to SEQ ID NOS: 401-428.

A modified IL-2 cytokine, comprising mutations at one or more amino acid residues of IL-2 corresponding to SEQ ID NO: 204: 43, 45, 48, 49, 52, 53, 60, 61, 65, 67, 68, 72, 100, 103, 104, 106, 107, 109, 110, and 132, wherein the mutations comprise insertions, deletions or  
20 replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 204 set forth in FIG12P and SEQ ID NOS: 429-476.

A modified IL-3 cytokine, comprising mutations at one or more  
25 amino acid residues of IL-3 corresponding to SEQ ID NO: 205: 37, 43, 46, 59, 63, 66, 96, 100, 101, and 103, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group

-98-

consisting of amino acid substitutions in SEQ ID NO:205 set forth in FIG12Q corresponding to SEQ ID NOS: 477-498.

A modified SCF cytokine, comprising mutations at one or more amino acid residues of SCF corresponding to SEQ ID NO: 206: 27, 31,  
5 34, 37, 54, 58, 61, 62, 63, 96, 98, 99, 100, 102, 103, 106, 107, 108, 109, 134, and 137, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 206 set forth in FIG12T  
10 corresponding to SEQ ID NOS: 499-542.

A modified IL-4 cytokine, comprising mutations at one or more amino acid residues of IL-4 corresponding to SEQ ID NO: 207: 26, 37, 53, 60, 61, 64, 66, 100, 102, 103, and 126, wherein the mutations comprise insertions, deletions or replacements of the native amino acid  
15 residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 207 set forth in FIG12R corresponding to SEQ ID NOS: 543-567.

A modified IL-5 cytokine, comprising mutations at one or more amino acid residues of IL-5 corresponding to SEQ ID NO: 208: 32, 34,  
20 39, 46, 47, 56, 84, 85, 88, 89, 90, 102, 110, and 111, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 208 set forth in FIG12S corresponding to SEQ ID NOS: 568-602.

25 A modified IL-13 cytokine, comprising mutations at one or more amino acid residues of IL-13 corresponding to SEQ ID NO: 209: 32, 34, 38, 48, 79, 82, 85, 86, 88, 107, 108, 110, and 111, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s). In particular embodiments, the replacements are

-99-

selected from the group consisting of amino acid substitutions in SEQ ID NO: 209 set forth in FIG12O corresponding to SEQ ID NOS: 603-630.

A modified G-CSF cytokine, comprising mutations at one or more amino acid residues of G-CSF corresponding to SEQ ID NO: 210: 61, 63,  
5 68, 72, 86, 96, 100, 101, 131, 133, 135, 147, 169, 172, and 177, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 210 set forth in FIG12E corresponding to  
10 SEQ ID NOS: 631-662.

A modified leptin cytokine, comprising mutations at one or more amino acid residues of leptin corresponding to SEQ ID NO: 211: 43, 49, 99, 100, 104, 105, 107, 108, 141 and 142, wherein the mutations comprise insertions, deletions or replacements of the native amino acid  
15 residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 211 set forth in FIG12I corresponding to SEQ ID NOS: 663-683.

A modified CNTF cytokine, comprising mutations at one or more amino acid residues of CNTF corresponding to SEQ ID NO: 212: 62, 64,  
20 66, 67, 86, 89, 92, 100, 102, 104, 131, 132, 133, 135, 136, 138, 140, 143, 148, and 151, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 212 set forth in FIG12D  
25 corresponding to SEQ ID NOS: 684-728.

A modified LIF cytokine, comprising mutations at one or more amino acid residues of LIF corresponding to SEQ ID NO: 213: 69, 70, 85, 99, 102, 104, 106, 109, 137, 143, 146, 148, 149, 153, 154, and 156, wherein the mutations comprise insertions, deletions or replacements of



-100-

the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 213 set forth in FIG12J corresponding to SEQ ID NOS: 729-760.

- 5           A modified oncostatin M cytokine, comprising mutations at one or more amino acid residues of oncostatin M corresponding to SEQ ID NO: 214: 59, 60, 63, 65, 84, 87, 89, 91, 94, 97, 99, 100, 103, and 106, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s). In particular embodiments, the
- 10 replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 214 set forth in FIG12K corresponding to SEQ ID NOS: 761-793.

- A modified IL-12 cytokine, comprising mutations at one or more amino acid residues of IL-12 corresponding to SEQ ID NO: 215: 56, 61,
- 15 66, 67, 68, 70, 72, 75, 78, 79, 82, 89, 92, 93, 107, 110, 111, 115, 117, 124, 125, 127, 128, 129, and 189, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 215 set
- 20 forth in FIG12G corresponding to SEQ ID NOS: 794-849.

- A modified hGH cytokine, comprising mutations at one or more amino acid residues of hGH corresponding to SEQ ID NO: 216: 56, 59, 64, 65, 66, 88, 92, 94, 101, 129, 130, 133, 134, 140, 143, 145, 146, 147, 183, and 186, wherein the mutations comprise insertions, deletions
- 25 or replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 216 set forth in FIG12F corresponding to SEQ ID NOS: 850-895.

-101-

A modified IL-6 cytokine, comprising mutations at one or more amino acid residues of IL-6 corresponding to SEQ ID NO: 217: 64, 65, 66, 68, 69, 75, 77, 92, 98, 103, 105, 108, 133, 138, 139, 140, 149, 156, 178, and 181, wherein the mutations comprise insertions, deletions  
5 or replacements of the native amino acid residue(s). In particular embodiments, the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 217 set forth in FIG12H corresponding to SEQ ID NOS: 896-939.

In certain embodiments, the modified cytokines provided herein  
10 possess increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity to either inhibit viral replication or to stimulate cell proliferation in appropriate cells, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

15 In other embodiments, the modified cytokines provided herein possess decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity to either inhibit viral replication in the appropriate cells or to stimulate cell proliferation of the appropriate cells, after incubation with either mixtures  
20 of proteases, individual proteases, blood lysate or serum.

In yet other embodiments, the modified cytokines provided herein possess increased biological activity compared to the unmodified cytokine, wherein activity is assessed by measuring the capacity to either inhibit viral replication in the appropriate cells or to stimulate cell  
25 proliferation of the appropriate cells, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

-102-

**H. Rational evolution of IFN $\beta$  for increased resistance to proteolysis and/or higher conformational stability**

Treatment with interferon b (IFN $\beta$ ) is a well established therapy. Typically it is used for treatment of multiple sclerosis (MS). Patients  
5 receiving interferon  $\beta$  are subject to frequent repeat applications of the drug. The instability of IFN $\beta$  in the blood stream and under the storage conditions is well known. Hence it would be useful to increasing stability (half-life) of IFN $\beta$  in serum and also *in vitro* would improve it as a drug.

The 2D-scanning method and the 3D-scanning method (using  
10 structural homology) provided herein (see, copending ) were each applied to interferon  $\beta$ . Provided herein are mutant variants of the IFN $\beta$  protein that display improved stability as assessed by resistance to proteases (thereby possessing increased protein half-life) and at least comparable biological activity as assessed by antiviral or antiproliferation activity  
15 compared to the unmodified and wild type native IFN $\beta$  protein (SEQ ID NO: 196). The IFN $\beta$  mutant proteins provided herein confer a higher half-life and at least comparable biological activity with respect to the native sequence. Thus, the optimized IFN $\beta$  protein mutants provided herein that possess increased resistance to proteolysis result in a decrease in the  
20 frequency of injections needed to maintain a sufficient drug level in serum, thus leading to, for example: *i*) higher comfort and acceptance by patients, *ii*) lower doses necessary to achieve comparable biological effects, and *iii*) as a consequence of (*ii*), likely attenuation of any secondary effects.

25 In particular embodiments, the half-life of the IFN $\beta$  mutants provided herein is increased by an amount selected from at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 150%, at least 200%, at least 250%, at least 300%, at least 350%, at least

-103-

400%, at least 450%, at least 500% or more, when compared to the half-life of native human IFN $\beta$  in either human blood, human serum or an in vitro mixture containing one or more proteases. In other embodiments, the half-life of the IFN $\beta$  mutants provided herein is increased by an  
5 amount selected from at least 6 times, 7 times, 8 times, 9 times, 10 times, 20 times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times, 100 times, 200 times, 300 times, 400 times, 500 times, 600 times, 700 times, 800 times, 900 times, 1000 times, or more, when compared to the half-life of native human IFN $\beta$  in either human blood,  
10 human serum or an in vitro mixture containing one or more proteases.

Two approaches were used herein to increase the stability of IFN $\beta$  by amino acid replacement: i) Resistance to proteases: amino acid replacement that leads to higher resistance to proteases by direct destruction of the protease target residue or sequence, while either  
15 maintaining or improving the requisite biological activity (e.g., antiviral and anti-proliferation activity), and/or ii) Conformational stability: amino acid replacement that leads to an increase in conformational stability (i.e. half-life at room temperature or at 37°C), while either improving or maintaining the requisite biological activity (e.g., antiviral and anti-  
20 proliferation activity).

Two methodologies were used to address the improvements described above: (a) 2D-scanning methods were used to identify aminoacid changes that lead to improvement in protease resistance and to improvement in conformational stability, and (b) 3D-scanning, which  
25 employs structural homology methods methods also were used to identify aminoacid changes that lead to improvement in protease resistance. The 2D-scanning and 3D-scanning methods each were used to identify the amino acid changes on IFN $\beta$  that lead to an increase in stability when challenged either with proteases, human blood lysate or human serum.

-104-

Increasing protein stability to proteases, human blood lysate or human serum is contemplated herein to provide a longer *in vivo* half-life for the particular protein molecules, and thus a reduction in the frequency of necessary injections into patients. The biological activities that have been

5 measured for the IFN $\beta$  molecules are *i*) their capacity to inhibit virus replication when added to permissive cells previously infected with the appropriate virus, and *ii*) their capacity to stimulate cell proliferation when added to the appropriate cells. Prior to the measurement of biological activity, IFN $\beta$  molecules were challenged with proteases, human blood

10 lysate or human serum during different incubation times. The biological activity measured, corresponds then to the residual biological activity following exposure to the proteolytic mixtures.

As set forth above, provided herein are methods for the generating IFN $\beta$  molecules (or any target protein, particularly cytokines) that, while

15 maintaining a requisite biological activity without substantial change (sufficient for therapeutic application(s)), have been rendered less susceptible to digestion by blood proteases and therefore display a longer half-life in blood circulation. In this particular example, the method used included the following specific steps as exemplified in the Examples:

20 For the improvement of resistance to proteases, by 2D-scanning, the method included:

- 1) Identifying some or all possible target sites on the protein sequence that are susceptible to digestion by one or more specific proteases (these sites are the is-HITs); and
- 25 2) Identifying appropriate replacing amino acids, specific for each is-HIT, such that if used to replace one or more of the original amino acids at that specific is-HIT, they can be expected to increase the is-HIT's resistance to digestion by protease while at the same time, keeping the

-105-

biological activity of the protein unchanged (these replacing amino acids are the candidate LEADs).

For the improvement of resistance to proteases, by 3D-scanning (structural homology):

- 5           1) Identifying some or all possible target sites (is-HITS) on the protein sequence that display an acceptable degree of structural homology around the aminoacid positions mutated in the LEAD molecules previously obtained for IFN $\alpha$  using 2D-scanning, and that are susceptible to digestion by one or more specific proteases; and
- 10          2) Identifying appropriate replacing amino acids, specific for each is-HIT, such that if used to replace one or more of the original amino acids at that specific is-HIT, they can be expected to increase the is-HIT's resistance to digestion by protease while at the same time, keeping the biological activity of the protein unchanged (these replacing amino acids
- 15 are the candidate LEADs).

For the improvement of conformational stability, by 2D-scanning, as provided herein:

- 20           1) Identifying some or all possible target sites on the protein sequence that are susceptible to being directly involved in the intramolecular flexibility and conformational change (these sites are the is-HITs); and
- 25           2) Identifying appropriate replacing amino acids, specific for each is-HIT, such that if used to replace one or more of the original amino acids at that specific is-HIT, they can be expected to increase the thermal stability of the molecule while at the same time, keeping the biological activity of the protein unchanged (these replacing amino acids are the candidate LEADs).

See Figures 6(O)-6(S) and Figure 8(A).

-106-

Using the 2D-scanning and 3D-scanning methods and the 3-dimensional structure of IFN $\beta$ , the following amino acid target positions were identified as is-HITs on IFN $\beta$ , which numbering is that of the mature protein (SEQ ID NO:196):

- 5           **By 3D-scanning** (see, SEQ ID Nos. 234-289, 989-1015): D by Q at position 39, D by H at position 39, D by G at position 39, E by Q at position 42, E by H at position 42, K by Q at position 45, K by T at position 45, K by S at position 45, K by H at position 45, L by V at position 47, L by I at position 47, L by T at position 47, L by Q at position 47, L by H at position 47, L by A at position 47, K by Q at position 52, K by T at position 52, K by S at position 52, K by H at position 52, F by I at position 67, F by V at position 67, R by H at position 71, R by Q at position 71, D by H at position 73, D by G at position 73, D by Q at position 73, E by Q at position 81, E by H at position 81, E by Q at position 107, E by H at position 107, K by Q at position 108, K by T at position 108, K by S at position 108, K by H at position 108, E by Q at position 109, E by H at position 109, D by Q at position 110, D by H at position 110, D by G at position 110, F by I at position 111, F by V at position 111, R by H at position 113, R by Q at position 113, L by V at position 116, L by I at position 116, L by T at position 116, L by Q at position 116, L by H at position 116, L by A at position 116, L by V at position 120, L by I at position 120, L by T at position 120, L by Q at position 120, L by H at position 120, L by A at position 120, K by Q at position 123, K by T at position 123, K by S at position 123, K by H at position 123, R by H at position 124,, R by Q at position 124, R by H at position 128, R by Q at position 128, L by V at position 130, L by I at position 130, L by T at position 130, L by Q at position 130, L by H at position 130, L by A at position 130, K by Q at position 134, K by T at position 134, K by S at position 134, K by H at

-107-

position 134, K by Q at position 136, K by T at position 136, K by S at position 136,, K by H at position 136, E by Q at position 137, E by H at position 137, Y by H at position 163, Y by I at position 163I, R by H at position 165, R by Q at position 165.

- 5 By 2D-scanning (see SEQ ID Nos.1016-1302) : M by V at position 1, M by I at position 1, M by T at position 1, M by Q at position 1 , M by A at position 1 , L by V at position 5 , L by I at position 5 , L by T at position 5 , L by Q at position 5 , L by H at position 5 , L by A at position 5 , F by I at position 8, F by V at position 8, L by V at position 9, L by I**
- 10 at position 9, L by T at position 9, L by Q at position 9, L by H at position 9, L by A at position 9, R by H at position 11, R by Q at position 11, F by I at position 15 , F by V at position 15 , K by Q at position 19, K by T at position 19, K by S at position 19, K by H at position 19, W by S at position 22, W by H at position 22, N by H at position 25, N by S at**
- 15 position 25, N by Q at position 25, R by H position 27, R by Q position 27, L by V at position 28, L by I at position 28, L by T at position 28, L by Q at position 28, L by H at position 28, L by A at position 28, E by Q at position 29, E by H at position 29, Y by H at position 30, Y by I at position 30, L by V at position 32, L by I at position 32, L by T at**
- 20 position 32, L by Q at position 32, L by H at position 32, L by A at position 32, K by Q at position 33, K by T at position 33, K by S at position 33, K by H at position 33, R by H at position 35, R by Q at position 35, M by V at position 36, M by I at position 36, M by T at position 36, M by Q at position 36, M by A at position 36, D by Q at**
- 25 position 39, D by H at position 39, D by G at position 39, E by Q at position 42, E by H at position 42, K by Q at position 45, K by T at position 45, K by S at position 45, K by H at position 45, L by V at position 47, L by I at position 47, L by T at position 47, L by, Q at position 47, L by H at position 47, L by A at position 47, K by Q at**



-108-

position 52, K by T at position 52, K by S at position 52, K by H at  
position 52, F by I at position 67, F by V at position 67, R by H at  
position 71, R by Q at position 71, D by Q at position 73, D by H at  
position 73, D by G at position 73, E by Q at position 81, E by H at  
5 position 81, E by Q at position 85, E by H at position 85, Y by H at  
position 92, Y by I at position 92, K by Q at position 99, K by T at  
position 99, K by S at position 99, K by H at position 99, E by Q at  
position 103, E by H at position 103, E by Q at position 104, E by H at  
position 104, K by Q at position 105, K by T at position 105, K by S at  
10 position 105, K by H at position 105, E by Q at position 107, E by H at  
position 107, K by Q at position 108, K by T at position 108, K by S at  
position 108, K by H at position 108, E by Q at position 109, E by H at  
position 109, D by Q at position 110, D by H at position 110, D by G at  
position 110, F by I at position 111, F by V at position 111, R by H at  
15 position 113, R by Q at position 113, L by V at position 116, L by I at  
position 116, L by T at position 116, L by Q at position 116, L by H at  
position 116, L by A at position 116, L by V at position 120, L by I at  
position 120, L by T at position 120, L by Q at position 120, L by H at  
position 120, L by A at position 120, K by Q at position 123, K by T at  
20 position 123, K by S at position 123, K by H at position 123, R by H at  
position 124, R by Q at position 124, R by H at position 128, R by Q at  
position 128, L by V at position 130, L by I at position 130, L by T at  
position 130, L by Q at position 130, L by H at position 130, L by A at  
position 130, K by Q at position 134, K by T at position 134, K by S at  
25 position 134, K by H at position 134, K by Q at position 136, K by T at  
position 136, K by S at position 136, K by H at position 136, E by Q at  
position 137, E by H at position 137, Y by H at position 138, Y by I at  
position 138, R by H at position 152, R by Q at position 152, Y by H at  
position 155, Y by I at position 155, R by H at position 159, R by Q at

-109-

position 159, Y by H at position 163, Y by I at position 163, R by H at  
position 165, R by Q at position 165, M by D at position 1, M by E at  
position 1, M by K at position 1, M by N at position 1, M by R at position  
1, M by S at position 1, L by D at position 5, L by E at position 5, L by K  
5 at position 5, L by N at position 5, L by R at position 5, L by S at position  
5, L by D at position 6, L by E at position 6, L by K at position 6, L by N  
at position 6, L by R at position 6, L by S at position 6, L by Q at position  
6, L by T at position 6, F by E at position 8, F by K at position 8, F by R  
at position 8, F by D at position 8, L by D at position 9, L by E at position  
10 9, L by K at position 9, L by N at position 9, L by R at position 9, L by S  
at position 9, Q by D at position 10, Q by E at position 10, Q by K at  
position 10, Q by N at position 10, Q by R at position 10, Q by S at  
position 10, Q by T at position 10, S by D at position 12, S by E at  
position 12, S by K at position 12, S by R at position 12, S by D at  
15 position 13, S by E at position 13, S by K at position 13, S by R at  
position 13, S by N at position 13, S by Q at position 13, S by T at  
position 13, N by D at position 14, N by E at position 14, N by K at  
position 14, N by Q at position 14, N by R at position 14, N by S at  
position 14, N by T at position 14, F by D at position 15, F by E at  
20 position 15, F by K at position 15, F by R at position 15, Q by D at  
position 16, Q by E at position 16, Q by K at position 16, Q by N at  
position 16, Q by R at position 16, Q by S at position 16, Q by T at  
position 16, C by D at position 17, C by E at position 17, C by K at  
position 17, C by N at position 17, C by Q at position 17, C by R at  
25 position 17, C by S at position 17, C by T at position 17, L by N at  
position 20, L by Q at position 20, L by R at position 20, L by S at  
position 20, L by T at position 20, L by D at position 20, L by E at  
position 20, L by K at position 20, W by D at position 22, W by E at  
position 22, W by K at position 22, W by R at position 22, Q by D at

-110-

position 23, Q by E at position 23, Q by K at position 23, Q by R at  
position 23, L by D at position 24, L by E at position 24, L by K at  
position 24, L by R at position 24, W by D at position 79, W by E at  
position 79, W by K at position 79, W by R at position 79, N by D at  
5 position 80, N by E at position 80, N by K at position 80, N by R at  
position 80, T by D at position 82, T by E at position 82, T by K at  
position 82, T by R at position 82, I by D at position 83, I by E at position  
83, I by K at position 83, I by R at position 83, I by N at position 83, I by  
Q at position 83, I by S at position 83, I by T at position 83, N by D at  
10 position 86, N by E at position 86, N by K at position 86, N by R at  
position 86, N by Q at position 86, N by S at position 86, N by T at  
position 86, L by D at position 87, L by E at position 87, L by K at  
position 87, L by R at position 87, L by N at position 87, L by Q at  
position 87, L by S at position 87, L by T at position 87, A by D at  
15 position 89, A by E at position 89, A by K at position 89, A by R at  
position 89, N by D at position 90, N by E at position 90, N by K at  
position 90, N by Q at position 90, N by R at position 90, N by S at  
position 90, N by T at position 90, V by D at position 91, V by E at  
position 91, V by K at position 91, V by N at position 91, V by Q at  
20 position 91, V by R at position 91, V by S at position 91, V by T at  
position 91, Q by D at position 94, Q by E at position 94, Q by Q at  
position 94, Q by N at position 94, Q by R at position 94, Q by S at  
position 94, Q by T at position 94, I by D at position 95, I by E at  
position 95, I by K at position 95, I by N at position 95, I by Q at position  
25 95, I by R at position 95, I by S at position 95, I by T at position 95, H by  
D at position 97, H by E at position 97, H by K at position 97, H by N at  
position 97, H by Q at position 97, H by R at position 97, H by S at  
position 97, H by T at position 97, L by D at position 98, L by E at  
position 98, L by K at position 98, L by N at position 98, L by Q at

-111-

position 98, L by R at position 98, L by S at position 98, L by T at position 98, V by D at position 101, V by E at position 101, V by K at position 101, V by N at position 101, V by Q at position 101, V by R at position 101, V by S at position 101, V by T at position 101, M by C at position 1, L by C at position 6, Q by C at position 10, S by C at position 13, Q by C at position 16, L by C at position 17, V by C at position 101, L by C at position 98, H by C at position 97, Q by C at position 94, V by C at position 91, N by C at position 90.

	SEQ ID NO.	Mutant
10	SEQ ID N° 1016	(M1V)
	SEQ ID N° 1017	(M1I)
	SEQ ID N° 1018	(M1T)
	SEQ ID N° 1019	(M1A)
	SEQ ID N° 1020	(L5V)
15	SEQ ID N° 1021	(L5I)
	SEQ ID N° 1022	(L5T)
	SEQ ID N° 1023	(L5Q)
	SEQ ID N° 1024	(L5H)
	SEQ ID N° 1025	(L5A)
20	SEQ ID N° 1026	(F8I)
	SEQ ID N° 1027	(F8V)
	SEQ ID N° 1028	(L9V)
	SEQ ID N° 1029	(L9I)
	SEQ ID N° 1030	(L9T)
25	SEQ ID N° 1031	(L9Q)
	SEQ ID N° 1032	(L9H)
	SEQ ID N° 1033	(L9A)
	SEQ ID N° 1034	(R11H)
	SEQ ID N° 1035	(R11Q)
30	SEQ ID N° 1036	(F15I)

-112-

	SEQ ID N° 1037	(F15V)
	SEQ ID N° 1038	(K19Q)
	SEQ ID N° 1039	(K19T)
	SEQ ID N° 1040	(K19S)
5	SEQ ID N° 1041	(K19H)
	SEQ ID N° 1042	(W22S)
	SEQ ID N° 1043	(W22H)
	SEQ ID N° 1044	(N25H)
	SEQ ID N° 1045	(N25S)
10	SEQ ID N° 1046	(N25Q)
	SEQ ID N° 1047	(R27H)
	SEQ ID N° 1048	(R27Q)
	SEQ ID N° 1049	(L28V)
	SEQ ID N° 1050	(L28I)
15	SEQ ID N° 1051	(L28T)
	SEQ ID N° 1052	(L28Q)
	SEQ ID N° 1053	(L28H)
	SEQ ID N° 1054	(L28A)
	SEQ ID N° 1055	(E29Q)
20	SEQ ID N° 1056	(E29H)
	SEQ ID N° 1057	(Y30H)
	SEQ ID N° 1058	(Y30I)
	SEQ ID N° 1059	(L32V)
	SEQ ID N° 1060	(L32I)
25	SEQ ID N° 1061	(L32T)
	SEQ ID N° 1062	(L32Q)
	SEQ ID N° 1063	(L32H)
	SEQ ID N° 1064	(L32A)
	SEQ ID N° 1065	(M1Q)
30	SEQ ID N° 1066	(K33Q)

-113-

	SEQ ID N° 1067	(K33T)
	SEQ ID N° 1068	(K33S)
	SEQ ID N° 1069	(K33H)
	SEQ ID N° 1070	(R35H)
5	SEQ ID N° 1071	(R35Q)
	SEQ ID N° 1072	(M36V)
	SEQ ID N° 1073	(M36I)
	SEQ ID N° 1074	(M36T)
	SEQ ID N° 1075	(M36Q)
10	SEQ ID N° 1076	(M36A)
	SEQ ID N° 1077	(E85Q)
	SEQ ID N° 1078	(E85H)
	SEQ ID N° 1079	(Y92H)
	SEQ ID N° 1080	(Y92I)
15	SEQ ID N° 1081	(K99Q)
	SEQ ID N° 1082	(K99T)
	SEQ ID N° 1083	(K99S)
	SEQ ID N° 1084	(K99H)
	SEQ ID N° 1085	(E103Q)
20	SEQ ID N° 1086	(E103H)
	SEQ ID N° 1087	(E104Q)
	SEQ ID N° 1088	(E104H)
	SEQ ID N° 1089	(K105Q)
	SEQ ID N° 1090	(K105T)
25	SEQ ID N° 1091	(K105S)
	SEQ ID N° 1092	(K105H)
	SEQ ID N° 1093	(Y138H)
	SEQ ID N° 1094	(Y138I)
	SEQ ID N° 1095	(R152H)
30	SEQ ID N° 1096	(R152Q)

-114-

	SEQ ID N° 1097	(Y155H)
	SEQ ID N° 1098	(Y155I)
	SEQ ID N° 1099	(R159H)
	SEQ ID N° 1100	(R159Q)
5	SEQ ID N° 1101	(M1D)
	SEQ ID N° 1102	(M1E)
	SEQ ID N° 1103	(M1K)
	SEQ ID N° 1104	(M1N)
	SEQ ID N° 1105	(M1R)
10	SEQ ID N° 1106	(M1S)
	SEQ ID N° 1107	(L5D)
	SEQ ID N° 1108	(L5E)
	SEQ ID N° 1109	(L5K)
	SEQ ID N° 1110	(L5R)
15	SEQ ID N° 1111	(L5N)
	SEQ ID N° 1112	(L5S)
	SEQ ID N° 1113	(L6D)
	SEQ ID N° 1114	(L6E)
	SEQ ID N° 1115	(L6K)
20	SEQ ID N° 1116	(L6N)
	SEQ ID N° 1117	(L6Q)
	SEQ ID N° 1118	(L6R)
	SEQ ID N° 1119	(L6S)
	SEQ ID N° 1120	(L6T)
25	SEQ ID N° 1121	(F8D)
	SEQ ID N° 1122	(F8E)
	SEQ ID N° 1123	(F8K)
	SEQ ID N° 1124	(F8R)
	SEQ ID N° 1125	(L9D)
30	SEQ ID N° 1126	(L9E)

-115-

	SEQ ID N° 1127	(L9K)
	SEQ ID N° 1128	(L9N)
	SEQ ID N° 1129	(L9R)
	SEQ ID N° 1130	(L9S)
5	SEQ ID N° 1131	(Q10D)
	SEQ ID N° 1132	(Q10E)
	SEQ ID N° 1133	(Q10K)
	SEQ ID N° 1134	(Q10N)
	SEQ ID N° 1135	(Q10R)
10	SEQ ID N° 1136	(Q10S)
	SEQ ID N° 1137	(Q10T)
	SEQ ID N° 1138	(S12D)
	SEQ ID N° 1139	(S12E)
	SEQ ID N° 1140	(S12K)
15	SEQ ID N° 1141	(S12R)
	SEQ ID N° 1142	(S13D)
	SEQ ID N° 1143	(S13E)
	SEQ ID N° 1144	(S13K)
	SEQ ID N° 1145	(S13N)
20	SEQ ID N° 1146	(S13Q)
	SEQ ID N° 1147	(S13R)
	SEQ ID N° 1148	(S13T)
	SEQ ID N° 1149	(N14D)
	SEQ ID N° 1150	(N14E)
25	SEQ ID N° 1151	(N14K)
	SEQ ID N° 1152	(N14Q)
	SEQ ID N° 1153	(N14R)
	SEQ ID N° 1154	(N14S)
	SEQ ID N° 1155	(N14T)
30	SEQ ID N° 1156	(F15D)



-116-

5	SEQ ID N° 1157	(F15E)
	SEQ ID N° 1158	(F15K)
	SEQ ID N° 1159	(F15R)
	SEQ ID N° 1160	(Q16D)
	SEQ ID N° 1161	(Q16E)
10	SEQ ID N° 1162	(Q16K)
	SEQ ID N° 1163	(Q16N)
	SEQ ID N° 1164	(Q16R)
	SEQ ID N° 1165	(Q16S)
	SEQ ID N° 1166	(Q16T)
15	SEQ ID N° 1167	(C17D)
	SEQ ID N° 1168	(C17E)
	SEQ ID N° 1169	(C17K)
	SEQ ID N° 1170	(C17N)
	SEQ ID N° 1171	(C17Q)
20	SEQ ID N° 1172	(C17R)
	SEQ ID N° 1173	(C17S)
	SEQ ID N° 1174	(C17T)
	SEQ ID N° 1175	(L20N)
	SEQ ID N° 1176	(L20Q)
25	SEQ ID N° 1177	(L20R)
	SEQ ID N° 1178	(L20S)
	SEQ ID N° 1179	(L20T)
	SEQ ID N° 1180	(L20D)
	SEQ ID N° 1181	(L20E)
30	SEQ ID N° 1182	(L20K)
	SEQ ID N° 1183	(W22D)
	SEQ ID N° 1184	(W22E)
	SEQ ID N° 1185	(W22K)
	SEQ ID N° 1186	(W22R)

-117-

5	SEQ ID N° 1187	(Q23D)
	SEQ ID N° 1188	(Q23E)
	SEQ ID N° 1189	(Q23K)
	SEQ ID N° 1190	(Q23R)
	SEQ ID N° 1191	(L24D)
10	SEQ ID N° 1192	(L24E)
	SEQ ID N° 1193	(L24K)
	SEQ ID N° 1194	(L24R)
	SEQ ID N° 1195	(G78D)
	SEQ ID N° 1196	(G78E)
15	SEQ ID N° 1197	(G78K)
	SEQ ID N° 1198	(G78R)
	SEQ ID N° 1199	(W79D)
	SEQ ID N° 1200	(W79E)
	SEQ ID N° 1201	(W79K)
20	SEQ ID N° 1202	(W79R)
	SEQ ID N° 1203	(N80D)
	SEQ ID N° 1204	(N80E)
	SEQ ID N° 1205	(N80K)
	SEQ ID N° 1206	(N80R)
25	SEQ ID N° 1207	(T82D)
	SEQ ID N° 1208	(T82E)
	SEQ ID N° 1209	(T82K)
	SEQ ID N° 1210	(T82R)
	SEQ ID N° 1211	(I83D)
30	SEQ ID N° 1212	(I83E)
	SEQ ID N° 1213	(I83K)
	SEQ ID N° 1214	(I83R)
	SEQ ID N° 1215	(I83N)
	SEQ ID N° 1216	(I83Q)

-118-

	SEQ ID N° 1217	(I83S)
	SEQ ID N° 1218	(I83T)
	SEQ ID N° 1219	(N86D)
	SEQ ID N° 1220	(N86E)
5	SEQ ID N° 1221	(N86K)
	SEQ ID N° 1222	(N86R)
	SEQ ID N° 1223	(N86Q)
	SEQ ID N° 1224	(N86S)
	SEQ ID N° 1225	(N86T)
10	SEQ ID N° 1226	(L87D)
	SEQ ID N° 1227	(L87E)
	SEQ ID N° 1228	(L87K)
	SEQ ID N° 1229	(L87R)
	SEQ ID N° 1230	(L87N)
15	SEQ ID N° 1231	(L87Q)
	SEQ ID N° 1232	(L87S)
	SEQ ID N° 1233	(L87T)
	SEQ ID N° 1234	(A89D)
	SEQ ID N° 1235	(A89E)
20	SEQ ID N° 1236	(A89K)
	SEQ ID N° 1237	(A89R)
	SEQ ID N° 1238	(N90D)
	SEQ ID N° 1239	(N90E)
	SEQ ID N° 1240	(N90K)
25	SEQ ID N° 1241	(N90Q)
	SEQ ID N° 1242	(N90R)
	SEQ ID N° 1243	(N90S)
	SEQ ID N° 1244	(N90T)
	SEQ ID N° 1245	(V91D)
30	SEQ ID N° 1246	(V91E)

-119-

	SEQ ID N° 1247	(V91K)
	SEQ ID N° 1248	(V91N)
	SEQ ID N° 1249	(V91Q)
	SEQ ID N° 1250	(V91R)
5	SEQ ID N° 1251	(V91S)
	SEQ ID N° 1252	(V91T)
	SEQ ID N° 1253	(Q94D)
	SEQ ID N° 1254	(Q94E)
	SEQ ID N° 1255	(Q94K)
10	SEQ ID N° 1256	(Q94N)
	SEQ ID N° 1257	(Q94R)
	SEQ ID N° 1258	(Q94S)
	SEQ ID N° 1259	(Q94T)
	SEQ ID N° 1260	(I95D)
15	SEQ ID N° 1261	(I95E)
	SEQ ID N° 1262	(I95K)
	SEQ ID N° 1263	(I95N)
	SEQ ID N° 1264	(I95Q)
	SEQ ID N° 1265	(I95R)
20	SEQ ID N° 1266	(I95S)
	SEQ ID N° 1267	(I95T)
	SEQ ID N° 1268	(H97D)
	SEQ ID N° 1269	(H97E)
	SEQ ID N° 1270	(H97K)
25	SEQ ID N° 1271	(H97N)
	SEQ ID N° 1272	(H97Q)
	SEQ ID N° 1273	(H97R)
	SEQ ID N° 1274	(H97S)
	SEQ ID N° 1275	(H97T)
30	SEQ ID N° 1276	(L98D)

-120-

	SEQ ID N° 1277	(L98E)
	SEQ ID N° 1278	(L98K)
	SEQ ID N° 1279	(L98N)
	SEQ ID N° 1280	(L98Q)
5	SEQ ID N° 1281	(L98R)
	SEQ ID N° 1282	(L98S)
	SEQ ID N° 1283	(L98T)
	SEQ ID N° 1284	(V101D)
	SEQ ID N° 1285	(V101E)
10	SEQ ID N° 1286	(V101K)
	SEQ ID N° 1287	(V101N)
	SEQ ID N° 1288	(V101Q)
	SEQ ID N° 1289	(V101R)
	SEQ ID N° 1290	(V101S)
15	SEQ ID N° 1291	(V101T)
	SEQ ID N° 1292	(M1C)
	SEQ ID N° 1293	(V101C)
	SEQ ID N° 1294	(L6C)
	SEQ ID N° 1295	(L98C)
20	SEQ ID N° 1296	(Q10C)
	SEQ ID N° 1297	(H97C)
	SEQ ID N° 1298	(S13C)
	SEQ ID N° 1299	(Q94C)
	SEQ ID N° 1300	(Q16C)
25	SEQ ID N° 1301	(N90C)
	SEQ ID N° 1302	(V91C)

-121-

**I. Super-LEADs and Additive Directional Mutagenesis (ADM)**

Also provided herein are super-LEAD mutant proteins comprising a combination of single amino acid mutations present in two or more of the respective LEAD mutant proteins. Thus, the super-LEAD mutant proteins

5 have two or more of the single amino acid mutations derived from two or more of the respective LEAD mutant proteins. As described herein, LEAD mutant proteins provided herein are defined as mutants whose performance or fitness has been optimized with respect to the native protein. LEADs typically contain one single mutation relative to its

10 respective native protein. This mutation represents an appropriate amino acid replacement that takes place at one is-HIT position. Further super-LEAD mutant proteins are created such that they carry on the same protein molecule, more than one LEAD mutation, each at a different is-HIT position. Once the LEAD mutant proteins have been identified using the

15 2D-scanning methods provided herein, super-LEADs can be generated by combining two or more individual LEAD mutant mutations using methods well-known in the art, such as recombination, mutagenesis and DNA shuffling, and by methods, such as additive directional mutagenesis and Multi-Overlapped Primer Extensions, provided herein.

**20 1) Additive Directional Mutagenesis.**

Also provided herein are methods for assembling on a single mutant protein multiple mutations present on the individual LEAD molecules, so as to generate super-LEAD mutant proteins. This method is referred to herein as "Additive Directional Mutagenesis" (ADM). ADM

25 comprises a repetitive multi-step process where at each step after the creation of the first LEAD mutant protein a new LEAD mutation is added onto the previous LEAD mutant protein to create successive super-LEAD mutant proteins. ADM is not based on genetic recombination mechanisms, nor on shuffling methodologies; instead it is a simple one-

-122-

mutation-at-a-time process, repeated as many times as necessary until the total number of desired mutations is introduced on the same molecule. To avoid the exponentially increasing number of all possible combinations that can be generated by putting together on the same molecule a given  
5 number of single mutations, a method is provided herein that, although it does not cover all the combinatorial possible space, still captures a big part of the combinatorial potential. The word "combinatorial" is used here in its mathematical meaning (i.e., subsets of a group of elements, containing some of the elements in any possible order) and not in the  
10 molecular biological or directed evolution meaning (i.e., generating pools, or mixtures, or collections of molecules by randomly mixing their constitutive elements).

A population of sets of nucleic acid molecules encoding a collection of new super-LEAD mutant molecules is generated, tested and  
15 phenotypically characterized one-by-one in addressable arrays. super-LEAD mutant molecules are such that each molecule contains a variable number and type of LEAD mutations. Those molecules displaying further improved fitness for the particular feature being evolved, are referred to as super-LEADs. Super-LEADs may be generated by other methods  
20 known to those of skill in the art and tested by the high throughput methods herein. For purposes herein a super-LEAD typically has activity with respect to the function or biological activity of interest that differs from the improved activity of a LEAD by a desired amount, such as at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%,  
25 150%, 200% or more from at least one of the LEAD mutants from which it is derived. In yet other embodiments, the change in activity is at least about 2 times, 3 times, 4 times, 5 times, 6 times, 7 times, 8 times, 9 times, 10 times, 20 times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times, 100 times, 200 times, 300 times, 400 times,

-123-

500 times, 600 times, 700 times, 800 times, 900 times, 1000 times, or more greater than at least one of the LEAD molecules from which it is derived. As with LEADs, the change in the activity for super-LEADs is dependent upon the activity that is being "evolved." The desired

5 alteration, which can be either an increase or a reduction in activity, will depend upon the function or property of interest.

In one embodiment provided herein, the ADM method employs a number of repetitive steps, such that at each step a new mutation is added on a given molecule. Although numerous different ways are

10 possible for combining each LEAD mutation onto a super-LEAD protein, an exemplary way the new mutations (e.g., mutation 1 (m1), mutation 2 (m2), mutation 3 (m3), mutation 4 (m4), mutation 5 (m5), mutation n (mn)) can be added corresponds to the following diagram:

	m1
15	m1 + m2
	m1 + m2 + m3
	m1 + m2 + m3 + m4
	m1 + m2 + m3 + m4 + m5
	m1 + m2 + m3 + m4 + m5 + ... + mn
20	m1 + m2 + m4
	m1 + m2 + m4 + m5
	m1 + m2 + m4 + m5 + ... + mn
	m1 + m2 + m5
	m1 + m2 + m5 + ... + mn
25	m2
	m2 + m3
	m2 + m3 + m4
	m2 + m3 + m4 + m5
	m2 + m3 + m4 + m5 + ... + mn



-124-

m2 + m4  
m2 + m4 + m5  
m2 + m4 + m5 + ... + mn  
m2 + m5  
5 m2 + m5 + ... + mn  
..., etc....

## 2) Multi-Overlapped Primer Extensions

In another embodiment, provided herein is a method for the rational evolution of proteins using oligonucleotide-mediated mutagenesis referred to as "multi overlapped primer extensions." This method can be used for the rational combination of mutant LEADs to form super-LEADS. This method allows the simultaneous introduction of several mutations throughout a small protein or protein-region of known sequence. Overlapping oligonucleotides of typically around 70 bases in length (since longer oligonucleotides lead to increased error) are designed from the DNA sequence (gene) encoding the mutant LEAD proteins in such a way that they overlap with each other on a region of typically around 20 bases. These overlapping oligonucleotides (including or not point mutations) act as both template and primers in a first step of PCR (using a proofreading polymerase, e.g., Pfu DNA polymerase, to avoid unexpected mutations) to create small amounts of full-length gene. The full-length gene resulting from the first PCR is then selectively amplified in a second step of PCR using flanking primers, each one tagged with a restriction site in order to facilitate subsequent cloning. One multi overlapped extension process yields a full-length (multi-mutated) nucleic acid molecule encoding a candidate super-LEAD protein having multiple mutations therein derived from LEAD mutant proteins.

Although typically about 70 bases are used to create the overlapping oligonucleotides, the length of additional overlapping

-125-

oligonucleotides for use herein can range from about 30 bases up to about 100 bases, from about 40 bases up to about 90 bases, from about 50 bases up to about 80 bases, from about 60 bases up to about 75 bases, and from about 65 bases up to about 75 bases. As set forth  
5 above, typically about 70 bases are used herein.

Likewise, although typically the overlapping region of the overlapping oligonucleotides is about 20 bases, the length of other overlapping regions for use herein can range from about 5 bases up to about 40 bases, from about 10 bases up to about 35 bases, from about  
10 15 bases up to about 35 bases, from about 15 bases up to about 25 bases, from about 16 bases up to about 24 bases, from about 17 bases up to about 23 bases, from about 18 bases up to about 22 bases, and from about 19 bases up to about 21 bases. As set forth above, typically about 20 bases are used herein for the overlapping region.

15 **J. Uses of the Mutant  $IFN\alpha$ ,  $IFN\beta$  Genes and Cytokines in Therapeutic Methods**

The optimized cytokines provided herein, such as the  $IFN\alpha$ -2b and  
15  $IFN\beta$  proteins and other modified cytokines, are intended for use in various therapeutic as well as diagnostic methods. These include all  
20 methods for which the unmodified proteins are used. By virtue of their improved phenotypes and activities, the proteins provided herein should exhibit improvement in the corresponding *in vivo* phenotype.

In particular, the optimized cytokines, such as the  $IFN\alpha$ -2b and  $IFN\beta$   
25 proteins, are intended for use in therapeutic methods in which cytokines have been used for treatment. Such methods include, but are not limited to, methods of treatment of infectious diseases, allergies, microbial diseases, pregnancy related diseases, bacterial diseases, heart diseases, viral diseases, histological diseases, genetic diseases, blood related diseases, fungal diseases, adrenal diseases, cancers, liver diseases,

-126-

autoimmune diseases, growth disorders, diabetes, neurodegenerative diseases, including multiple sclerosis, Parkinson's disease and Alzheimer's disease.

### 1) Fusion Proteins

5 Fusion proteins containing a targeting agent and mutant IFN $\alpha$ , including IFN $\alpha$ -2b and IFN $\alpha$ -2a, and IFN $\beta$  mutant proteins, or cytokine protein also are provided. Pharmaceutical compositions containing such fusion proteins formulated for administration by a suitable route are provided. Fusion proteins are formed by linking in any order the mutant  
10 protein and an agent, such as an antibody or fragment thereof, growth factor, receptor, ligand and other such agent for directing the mutant protein to a targeted cell or tissue. Linkage can be effected directly or indirectly via a linker. The fusion proteins can be produced recombinantly or chemically by chemical linkage, such as via heterobifunctional agents  
15 or thiol linkages or other such linkages. The fusion proteins can contain additional components, such as *E. coli* maltose binding protein (MBP) that aid in uptake of the protein by cells (see, International PCT application No. WO 01/32711).

### 2) Nucleic Acid Molecules for Expression

20 Nucleic acid molecules encoding the mutant cytokines including the mutant IFN $\beta$  proteins and IFN  $\alpha$  proteins, such as the IFN $\alpha$ -2b and IFN $\alpha$ -2a proteins, provided herein, or the fusion protein operably linked to a promoter, such as an inducible promoter for expression in mammalian cells also are provided. Such promoters include, but are not limited to,  
25 CMV and SV40 promoters; adenovirus promoters, such as the E2 gene promoter, which is responsive to the HPV E7 oncoprotein; a PV promoter, such as the PBV p89 promoter that is responsive to the PV E2 protein; and other promoters that are activated by the HIV or PV or oncogenes.

-127-

The mutant cytokines including the mutant interferons (IFN $\alpha$ 's and IFN $\beta$ ) proteins provided herein, also can be delivered to the cells in gene transfer vectors. The transfer vectors also can encode additional other therapeutic agent(s) for treatment of the disease or disorder, such as cancer or HIV infection, for which the cytokine is administered.

### 3) Formulation of Optimized Cytokines

Pharmaceutical compositions containing an optimized cytokine produced herein, such as IFN $\alpha$ -2b, IFN $\alpha$ -2a and IFN $\beta$ , fusion proteins or encoding nucleic acid molecules can be formulated in any conventional manner by mixing a selected amount of an optimized cytokine with one or more physiologically acceptable carriers or excipients. Selection of the carrier or excipient depends upon the mode of administration (*i.e.*, systemic, local, topical or any other mode) and disorder treated. The pharmaceutical compositions provided herein can be formulated for single dosage administration. The concentrations of the compounds in the formulations are effective for delivery of an amount, upon administration, that is effective for the intended treatment. Typically, the compositions are formulated for single dosage administration. To formulate a composition, the weight fraction of a compound or mixture thereof is dissolved, suspended, dispersed or otherwise mixed in a selected vehicle at an effective concentration such that the treated condition is relieved or ameliorated. Pharmaceutical carriers or vehicles suitable for administration of the compounds provided herein include any such carriers known to those skilled in the art to be suitable for the particular mode of administration.

In addition, the compounds may be formulated as the sole pharmaceutically active ingredient in the composition or may be combined with other active ingredients. Liposomal suspensions, including tissue-targeted liposomes, may also be suitable as pharmaceutically acceptable

-128-

carriers. These may be prepared according to methods known to those skilled in the art. For example, liposome formulations may be prepared as described in U.S. Patent No. 4,522,811.

The active compound is included in the pharmaceutically acceptable carrier in an amount sufficient to exert a therapeutically useful effect in the absence of undesirable side effects on the patient treated. The therapeutically effective concentration may be determined empirically by testing the compounds in known *in vitro* and *in vivo* systems, such as the assays provided herein. The active compounds can be administered by any appropriate route, for example, orally, parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid, semi-liquid or solid form and are formulated in a manner suitable for each route of administration.

The optimized cytokine and physiologically acceptable salts and solvates can be formulated for administration by inhalation (either through the mouth or the nose) or for oral, buccal, parenteral or rectal administration. For administration by inhalation, the optimized cytokine can be delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluorethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit can be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g., gelatin for use in an inhaler or insufflator can be formulated containing a powder mix of a therapeutic compound and a suitable powder base such as lactose or starch.

For oral administration, the pharmaceutical compositions can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding

-129-

agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets can be coated by methods well known in the art. Liquid preparations for oral administration can take the form of, for example, solutions, syrups or suspensions, or they can be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations can also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration can be suitably formulated to give controlled release of the active compound. For buccal administration the compositions can take the form of tablets or lozenges formulated in conventional manner.

The optimized cytokine can be formulated for parenteral administration by injection e.g., by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active

-130-

ingredient can be in powder-lyophilized form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

In addition to the formulations described previously, the optimized cytokine also can be formulated as a depot preparation. Such long acting  
5 formulations can be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the therapeutic compounds can be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives,  
10 for example, as a sparingly soluble salt.

The active agents can be formulated for local or topical application, such as for topical application to the skin and mucous membranes, such as in the eye, in the form of gels, creams, and lotions and for application to the eye or for intracisternal or intraspinal application. Such solutions,  
15 particularly those intended for ophthalmic use, can be formulated as 0.01% - 10% isotonic solutions, pH about 5-7, with appropriate salts. The compounds can be formulated as aerosols for topical application, such as by inhalation (see, e.g., U.S. Patent Nos. 4,044,126, 4,414,209, and 4,364,923, which describe aerosols for delivery of a steroid useful  
20 for treatment inflammatory diseases, particularly asthma).

The concentration of active compound in the drug composition will depend on absorption, inactivation and excretion rates of the active compound, the dosage schedule, and amount administered as well as other factors known to those of skill in the art. For example, the amount  
25 that is delivered is sufficient to treat the symptoms of hypertension.

The compositions may, if desired, be presented in a pack or dispenser device which can contain one or more unit dosage forms containing the active ingredient. The pack can for example, comprise

-131-

metal or plastic foil, such as a blister pack. The pack or dispenser device can be accompanied by instructions for administration.

The active agents can be packaged as articles of manufacture containing packaging material, an agent provided herein, and a label that  
5 indicates the disorder for which the agent is provided.

#### K. Examples

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention. The specific methods exemplified can be practiced with other species. The examples  
10 are intended to exemplify generic processes.

##### EXAMPLE 1

This example describes a plurality of chronological steps including steps from (i) to (viii):

- 15 (i) cloning of IFN $\alpha$  cDNA in a mammalian cell expression plasmid (section A.1)
- (ii) generation of a collection of targeted mutants on the IFN $\alpha$  cDNA in the mammalian cell expression plasmid (section B)
- (iii) production of IFN $\alpha$  mutants in mammalian cells (section C.1)
- (iv) screening and partial in vitro characterization of IFN $\alpha$  mutants  
20 produced in mammalian cells in search of lead mutants (section D)
- (v) cloning of the lead mutants into a bacterial cell expression plasmid (section A.2)
- (vi) expression of lead mutants in bacterial cells (section C.2)
- (vii) *in vitro* characterization of lead mutants produced in bacteria  
25 (section D)
- (viii) in vivo characterization of lead mutants produced in bacteria (section E).



-132-

**A. Cloning of IFN $\alpha$ -2b encoding cDNA****A.1. Cloning of IFN  $\alpha$ -2b cDNA in a mammalian cell expression plasmid**

5 The IFN  $\alpha$ -2b cDNA was first cloned into an mammalian expression vector, prior to the generation of the selected mutations. A collection of mutants was then generated such that each individual mutant was created and processed individually, physically separated from each other and in addressable arrays. The mammalian expression vector pSSV9 CMV 0.3 pA was engineered as follows:

10 The pSSV9 CMV 0.3 pA was cut by *PvuII* and religated (this step gets rid of the ITR functions), prior to the introduction of a new *EcoRI* restriction site by Quickchange mutagenesis (Stratagene). The oligonucleotides primers were:

EcoRI forward primer 5'-  
15 GCCTGTATGATTTATTGGATGTTGGAATTCC-  
CTGATGCGGTATTTTCTCCTTACG-3' (SEQ ID NO: 218)  
EcoRI reverse primer 5'-  
CGTAAGGAGAAAATACCGCATCAGGGAATT-  
CCAACATCCAATAAATCATACAGGC-3' (SEQ ID NO: 219).

20 The construct sequence was confirmed by using the following oligonucleotides:

Seq *Clal* forward primer: 5'-CTGATTATCAACCGGGGTACATATGATTGAC-  
ATGC-3' (SEQ ID NO: 220)

Seq *XmnI* reverse primer 5'-TACGGGATAATACCGCGCCACATAGCAGAA-C-3'  
25 (SEQ ID NO: 221).

Then, the *XmnI*-*Clal* fragment containing the newly introduced *EcoRI* site was cloned into pSSV9 CMV 0.3 pA (SSV9 is a clone containing the entire adeno-associated virus (AAV) genome inserted into the *PvuII* site of plasmid pEMBL (see, Du *et al.* (1996) *Gene Ther*

-133-

3:254-261)) to replace the corresponding wild-type fragment and produce construct pSSV9-2EcoRI.

The DNA sequence of the IFN $\alpha$ -2b cDNA, which was inserted into the mammalian vector pDG6 (ATCC accession No. 53169), was

5 confirmed using a pair of internal primers. The sequences of the IFN $\alpha$ -2b-related oligonucleotides for sequencing follow:

Seq forward primer: 5'-CCTGATGAAGGAGGACTC-3' (SEQ ID NO: 222)

Seq reverse primer: 5'-CCAAGCAGCAGATGAGTC-3' (SEQ ID NO: 223).

10 Since the beginning of the IFN $\alpha$ -2b encoding cDNA (the signal peptide encoding sequence) is absent in pDG6, it was added using the oligonucleotide (see below) to the amplified gene. First, the IFN $\alpha$ -2b cDNA was amplified by PCR using pDG6 as template using the following oligonucleotides as primers:

15 IFN $\alpha$ -2b 5' primer 5'-TCAGCTGCAAGTCAAGCTGCTCTGTGGGCTG-3' (SEQ ID NO: 224)

IFN $\alpha$ -2b 3' primer 5'-GCTCTAGATCATTCTTACTTCTTAACTTTC-TTGCAAGTTTGTGAC-3' (SEQ ID NO: 225)

20 The PCR product was then used in an overlapping PCR using the following oligonucleotide sequences, having *Hind* III or *Xba*I restriction sites (underlined) or the DNA sequence missing in pDG6 (underlined):

IFN $\alpha$ -2b *Hind*III primer 5'-CCCAAGCTTATTGGCCTTGACCTTTGCTTTACT-GGTG-3' (SEQ ID NO: 226)

IFN $\alpha$ -2b *Xba*I primer 5'-GCTCTAGATCATTCTTACTTCTTAACTTTC-TTGCAAGTTTGTGAC-3' (SEQ ID NO: 227)

25 IFN $\alpha$ -2b 80bp 5' primer 5'-CCCAAGCTTATGGCCTTGACCTTTGCTTACTGGTGGCCCTCCTGGTGCTCAGCTGCAAGTCAAGCTGCTCTGTGGGCTG-3' (SEQ ID NO: 228).

The entire IFN $\alpha$ -2b cDNA was cloned into the pTOPO-TA vector (Invitrogen). After checking gene sequence by automatic DNA  
30 sequencing, the *Hind*III-*Xba*I fragment containing the gene of interest was

-134-

subcloned into the corresponding sites of pSSV9-2EcoRI to produce pAAV-EcoRI-IFN $\alpha$ -2b (pNB-AAV-IFN  $\alpha$ -2b).

## A.2 Cloning of the IFN $\alpha$ -2b leads in an *E. coli* expression plasmid

### A.2.1 Characterization of the bacterial cells

- 5 BL21-CodonPlus(DE3)-RP<sup>®</sup> competent *Escherichia coli* cells are derived from Stratagene's high-performance BL21-Gold competent cells. These cells enable efficient high-level expression of heterologous proteins in *E. coli*. Efficient production of heterologous proteins in *E. coli* is frequently limited by the rarity, in *E. coli*, of certain tRNAs that are
- 10 abundant in the organisms from which the heterologous proteins are derived. Availability of tRNAs allows high-level expression of many heterologous recombinant genes in BL21-Codon Plus cells that are poorly expressed in conventional BL21 strains. BL21-CodonPlus(DE3)-RP cells contain a ColE1-compatible, pACYC-based plasmid containing extra
- 15 copies of the *argU* and *proL* tRNA genes.

### A.2.2 Cloning of wild-type IFN $\alpha$

- To express IFN  $\alpha$ -2b in *E. coli* cDNA encoding the mature form of IFN-2  $\alpha$ -2b was finally cloned into the plasmid pET-11 (Novagen). Briefly, this cDNA fragment was amplified by PCR using the primers SEQ ID Nos.
- 20 1306 and 1305, respectively:
- FOR-IFNA-5' AACATATGTGTGATCTGCCTCAAACCCACAGCCTGGGTAGC 3'  
REV-IFNA-5' AAGGATCCTCATTCCTTACTTCTTAACTTTCTTGCAAGTTTGTG3',
- from pSSV9-EcoRI-IFN  $\alpha$ -2b (see above), which contains full-length IFN-2  $\alpha$  cDNA as a matrix, using Herculase DNA-polymerase (Stratagene).
- 25 The PCR fragment was subcloned into pTOPO-TA vector (Invitrogen) yielding pTOPO-IFN  $\alpha$ -2b. The sequence was verified by sequencing. pET11 IFN  $\alpha$ -2b was prepared by insertion of the *NdeI*-*Bam* *HI* (Biolabs) fragment from pTOPO-IFN  $\alpha$ -2b into the *NdeI*-*Bam* *HI* sites of pET 11. The

-135-

DNA sequence of the resulting pET 11-IFN  $\alpha$ -2b construct was verified by sequencing and the plasmid was used for IFN  $\alpha$ -2b expression in *E.coli*.

**A.2.3 Cloning of IFN  $\alpha$ -2b mutants from the mammalian expression plasmid into the E.coli expression plasmid**

- 5       Lead mutants of Interferon alpha were first generated in the pSSV9-IFNa-EcoRI plasmid. With the only exception of E159H and E159Q, all mutants were amplified using the primers below. Primers contained NdeI (in Forward) and BamHI (in Reverse) restriction sites: FOR-IFNA-5' AACATATGTGTGATCTGCCTCAAACCCACAGCCTGGGTAGC 3' (SEQ ID
- 10 No. 1306; and REV-IFNA-5' AAGGATCCTCATTCCTTACTTCTTAACTTTCTTGCAAGTTTGTTG 3' (SEQ ID No. 1305)
- Mutants E159H and E159Q were amplified using the following primers on reverse side (primer forward was the same than described above):
- 15 REV-IFNA-E159H-5' AAGGATCCTCATTCCTTACTTCTTAACTGTGTTGCAAGTTTGTTG 3' SEQ ID No. 1304 above; and REV-IFNA-E159Q-5' AAGGATCCTCATTCCTTACTTCTTAACTCTGTTGCAAGTTTGTTG 3' SEQ ID No. 1305.
- Mutants were amplified with Pfu Turbo Polymerase (Stratagene)
- 20 according. PCR products were cloned into pTOPO plasmid (Zero Blunt TOPO PCR cloning kit, Invitrogen). The presence of the desired mutations was checked by automatic sequencing. The NdeI + BamHI fragment of the pTOPO-IFNa positive clones was then cloned into NdeI + BamHI sites of the pET11 plasmid.
- 25 **B. Construction of a collection of IFN $\alpha$ -2b mutants in a mammalian expression plasmid**
- A series of mutagenic primers was designed to generate the appropriate site-specific mutations in the IFN $\alpha$ -2b cDNA. Mutagenesis reactions were performed with the Chameleon<sup>®</sup> mutagenesis kit
- 30 (Stratagene) using pNB-AAV-IFN $\alpha$ -2b as the template. Each individual mutagenesis reaction was designed to generate one single mutant protein.

-136-

Each individual mutagenesis reaction contains one and only one mutagenic primer. For each reaction, 25 pmoles of each (phosphorylated) mutagenic primer were mixed with 0.25 pmoles of template, 25 pmoles of selection primer (introducing a new restriction site), and 2  $\mu$ l of 10X

5 mutagenesis buffer (100 mM Tris-acetate pH 7.5; 100 mM MgOAc; 500 mM KOAc pH 7.5) into each well of 96 well-plates. To allow DNA annealing, PCR plates were incubated at 98 °C during 5 min and immediately placed 5 min on ice, before incubating at room temperature during 30 min. Elongation and ligation reactions were allowed by addition

10 of 7  $\mu$ l of nucleotide mix (2.86 mM each nucleotide; 1.43 X mutagenesis buffer) and 3  $\mu$ l of a freshly prepared enzyme mixture of dilution buffer (20 mM Tris HCl pH7.5; 10 mM KCl; 10 mM  $\beta$ -mercaptoethanol; 1 mM DTT; 0.1 mM EDTA; 50 % glycerol), native T7 DNA polymerase (0.025 U/ $\mu$ l), and T4 DNA ligase (1 U/ $\mu$ l) in a ratio of 1:10, respectively.

15 Reactions were incubated at 37 °C for 1 h before inactivation of T4 DNA ligase at 72 °C during 15 min. In order to eliminate the parental plasmid, 30  $\mu$ l of a mixture containing 1X enzyme buffer and 10 U of restriction enzyme was added to the mutagenic reactions followed by incubation at 37 °C for at least 3 hours. Next, 90  $\mu$ l aliquots of *XLmutS* competent

20 cells (Stratagene) containing 25 mM  $\beta$ -mercaptoethanol were placed in ice-chilled deep-well plates. Then, plates were incubated on ice for 10 min with gentle vortex every 2 min. Transformation of competent cells was performed by adding aliquots of the restriction reactions (1/10 of reaction volume) and incubating on ice for 30 min. A heat pulse was performed in

25 a 42 °C water bath for 45 s, followed by incubation on ice for 2 minutes. Preheated SOC medium (0.45 ml) was added to each well and plates were incubated at 37 °C for 1 h with shaking. In order to enrich for mutated plasmids, 1 ml of 2 X YT broth medium supplemented with 100  $\mu$ g/ml ampicillin was added to each transformation mixture followed by

-137-

overnight incubation at 37 °C with shaking. Plasmid DNA isolation was performed by alkaline lysis using Nucleospin Multi-96 Plus Plasmid Kit (Macherey-Nagel) according to the manufacturer's instructions. Selection of mutated plasmids was performed by digesting 500 µg of plasmid preparation with 10 U of selection endonuclease in an overnight incubation at 37 °C. A fraction of the digested reactions (1/10 of the total volume) was transformed into 40 µl of Epicurian coli XL1-Blue competent cells (Stratagene) supplemented with 25 mM β-mercaptoethanol.

Transformation was performed as described above. Transformants were selected on LB-ampicillin agar plates incubated overnight at 37 °C. Isolated colonies were picked up and grown overnight at 37 °C into deep-well plates. Four clones per reaction were screened by endonuclease digestion of a new restriction site introduced by the selection primer. Finally, each mutation that was introduced to produce this collection of candidate LEAD IFNα-2b mutant plasmids encoding the proteins set forth in Table 2 of Example 2 was confirmed by automatic DNA sequencing.

#### C. Production of IFNα-2b mutants

##### C.1 In mammalian cells

IFNα-2b mutants were produced in 293 human embryo kidney (HEK) cells (obtained from ATCC), using Dubelcco's modified Eagle's medium supplemented with glucose (4.5 g/L; Gibco-BRL) and fetal bovine serum (10%, Hyclone). Cells were transiently transfected with the plasmids encoding the IFNα-2b mutants as follows:  $0.6 \times 10^5$  cells were seeded into 6 well-plates and grown for 36 h before transfection. Confluent cells at about 70%, were supplemented with 2.5 µg of plasmid (IFNα-2b mutants) and 10 mM poly-ethylene-imine (25 KDa PEI, Sigma-Aldrich). After gently shaking, cells were incubated for 16 h. Then, the

-138-

culture medium was changed with 1 ml of fresh medium supplemented with 1% of serum. IFN $\alpha$ -2b was measured on culture supernatants obtained 40 h after transfection and stored in aliquots at -80 °C until use.

Supernatants containing IFN $\alpha$ -2b from transfected cells were  
5 screened following sequential biological assays as follows. Normalization of IFN $\alpha$ -2b concentration from culture supernatants was performed by enzyme-linked immunoabsorbent assay (ELISA) using a commercial kit (R & D) and following the manufacturer's instructions. This assay includes plates coated with an IFN $\alpha$ -2b monoclonal antibody that can be developed  
10 by coupling a secondary antibody conjugated to the horseradish peroxidase (HRP). IFN $\alpha$ -2b concentrations on samples containing (i) wild type IFN $\alpha$ -2b produced under comparable conditions as the mutants, (ii) the IFN $\alpha$ -2b mutants and (iii) control samples (produced from cells expressing GFP) were estimated by using an international reference  
15 standard provided by the NIBSC, UK.

#### C.2 In bacteria

A volume of 200 ml of culture medium (LB/Ampicillin/  
Chloramphenicol) was inoculated with 5 ml of pre-culture BL21-  
pCodon + -pET-IFN  $\alpha$ -2b muta overnight at 37 °C with constant shaking  
20 (225 rpm). The production of IFN  $\alpha$ -2b was induced by the addition of 50  $\mu$ l of 2M IPTG at DO<sub>600nm</sub> ~ 0.6.

The culture was continued for 3 additional hours and was centrifuged at 4°C and 5000 g for 15 minutes. The supernatant (culture medium) was discarded and bacteria were lysed in 8 ml of lysis buffer by  
25 thermal shock (freezing – thawing: 37°C – 15 min; -80°C – 10 min; 37°C – 15 min; -80°C – 10 min; 37°C – 15 min). After centrifugation (10000 g, 15 min, 4°C), the supernatant (soluble proteins fraction) was discarded, and the precipitated material (insoluble protein fraction containing the IFN  $\alpha$  -2b protein as inclusion bodies) was purified.

-139-

### **C.3 Pre-purification of IFN $\alpha$ -2b as inclusion bodies in *E. coli***

#### **C.3.1 Washing of inclusion bodies by sonication**

Pellets containing the inclusion bodies were suspended in 10 ml of buffer and sonicated (80 watts) on ice, 1 second "on," 1 second "off" for  
5 a total of 4 min. Suspensions were then centrifuged (4°C, 10000 g, 15 min), and supernatants were recovered. Pellets were resuspended in 10 ml of buffer for a new sonication/centrifugation cycle. Triton X-100 was then eliminated by two additional cycles of sonication/centrifugation with buffer. Pellets containing the inclusion bodies were recovered and  
10 dissolved. The washed supernatants were stored at 4°C.

#### **C.3.2 Solubilization of inclusion bodies by denaturation**

Once washed, the inclusion bodies were solubilized in buffer at a concentration estimated in 0.3 mg/ml measuring the OD<sub>280</sub> (considering the coefficient of molar extinction of IFN  $\alpha$ -2b). Solubilization was carried  
15 out overnight at 4°C, under shaking.

#### **C.3.3 Renaturation of IFN $\alpha$ -2b by dialysis of GdnHCl**

Samples contained 1 mg of protein at 0.3 mg/ml (5 ml in total) in buffer. The GdnHCl (Hydrochloride Guanidium) present in the samples was eliminated by dialysis (minimum membrane cut = 10 kDa) overnight  
20 at 4°C against buffer (1litre) (final concentration of GdnHCl : 43 Mm). Next, samples were further dialysed against 1litre of buffer during 2:30h. This step was repeated two additional times. After dialysis, very little precipitate was visible.

### **D. Screening and *in vitro* characterization of IFN $\alpha$ -2b mutants**

25 Two activities were measured directly on IFN samples: antiviral and antiproliferation activities. Dose (concentration) - response (activity) experiments for antiviral or antiproliferation activity permitted calculation of the 'póteny' for antiviral and antiproliferation activities, respectively. Antiviral and antiproliferation activities also were measured after



-140-

incubation with proteolytic samples, such as specific proteases, mixtures of selected proteases, human serum or human blood. Assessment of activity following incubation with proteolytic samples allowed to determine the residual (antiviral or antiproliferation) activity and the  
5 respective kinetics of half-life upon exposure to proteases.

#### **D.1. Antiviral activity**

IFN $\alpha$ -2b protects cells against viral infection by a complex mechanism devoted to create an unfavorable environment for viral proliferation. Cellular antiviral response due to IFN $\alpha$ -2b (IFN anti-viral  
10 assay) was assessed using an interferon-sensitive HeLa cell line (ATCC accession no. CCL-2) treated with the encephalomyocarditis virus (EMCV). The assessment of either the virus-induced cytopathic effects (CPE) or the amount of EMCV mRNA in extracts of infected cells by RT-PCR was used to determine IFN $\alpha$  activity in samples.

##### **15 D.1.1 Antiviral activity - measure by RT-qPCR**

Confluent cells were trypsinized and plated at density  $2 \times 10^4$  cells/well in DMEM 5% SVF medium (Day 0). Cells were incubated with IFN  $\alpha$ -2b (at a concentration of 500 U/ml) to get 500 pg/ml and 150 pg/well (100  $\mu$ l of IFN solution), during 24 h at 37 °C prior to be  
20 challenged with EMCV (1/1000 dilution; MOI 100). After an incubation of 16 h, when virus-induced CPE was near maximum in untreated cells, the number of EMCV particles in each well was determined by RT-PCR quantification of EMCV mRNA, using lysates of infected cells. RNA from cell extracts was purified after a DNase/proteinase K treatment (Applied  
25 Biosystems). The CPE was evaluated using both Uptibleu (Interchim) and MTS (Promega) methods, which are based on detecting bio-reductions produced by the metabolic activity of cells in a flourometric and colorimetric manner, respectively. In order to produce a standard curve for EMCV quantification, a 22 bp DNA fragment of the capsid protein-

-141-

cDNA was amplified by PCR and cloned into pTOPO-TA vector (Invitrogen). Next, RT-PCR quantification of known amounts of pTOPO-TA-EMCV capsid gene was performed using the One-step RT-PCR kit (Applied Biosystems) and the following EMCV-related (cloning)

5 oligonucleotides and probe:

EMCV forward primer 5'-CCCCTACATTGAGGCATCCA-3' (SEQ ID NO: 229)

EMCV reverse primer 5'-CAGGAGCAGGACAAGGTCACT-3' (SEQ ID NO: 230)

EMCV probe 5'-(FAM)CAGCCGTCAAGACCCAACCGCT(TAMR A)-3' (SEQ ID NO: 231).

10 **D.1.2 Antiviral activity - measure by CPE**

Antiviral activity of IFN  $\alpha$ -2b was determined by the capacity of the cytokine to protect Hela cells against EMC (mouse encephalomyocarditis) virus-induced cytopathic effects. The day before, Hela cells ( $2 \times 10^5$  cells/ml) were seeded in flat-bottomed 96-well plates containing 100

15  $\mu$ l/well of Dulbecco's MEM-Glutamax1-sodium pyruvate medium supplemented with 5% SVF and 0.2% of gentamicin. Cells were growth at 37°C in an atmosphere of 5% CO<sub>2</sub> for 24 hours.

Two-fold serial dilutions of interferon samples were made with MEM complete media into 96-Deep-Well plates with final concentration  
20 ranging from 1600 to 0.6 pg/ml. The medium was aspirated from each well and 100  $\mu$ l of interferon dilutions were added to Hela cells. Each interferon sample dilution was assessed in triplicate. The two last rows of the plates were filled with 100  $\mu$ l of medium without interferon dilution samples in order to serve as controls for cells with and without virus.

25 After 24 hours of growth, a 1/1000 EMC virus dilution solution was placed in each well except for the cell control row. Plates were returned to the CO<sub>2</sub> incubator for 48 hours. Then, the medium was aspirated and the cells were stained for 1 hour with 100  $\mu$ l of Blue staining solutio to determine the proportion of intact cells. Plates were  
30 washed in a distilled water bath. The cell bound dye was extracted using

-142-

100  $\mu$ l of ethylene-glycol mono-ethyl-ether (Sigma). The absorbance of the dye was measured using an Elisa plate reader (Spectramax). The antiviral activity of IFN  $\alpha$ -2b samples (expressed as number of IU/mg of proteins) was determined as the concentration needed for 50% protection  
5 of the cells against EMC virus-induced cytopathic effects. For proteolysis experiments, each point of for the kinetic measurements was assessed at 500 and 166 pg/ml in triplicate.

#### D.2 Antiproliferation activity

Anti-proliferative activity of interferon  $\alpha$ -2b was determined by the  
10 capacity of the cytokine to inhibit proliferation of Daudi cells. Daudi cells ( $1 \times 10^4$  cells) were seeded in flat-bottomed 96-well plates containing 50  $\mu$ l/well of RPMI 1640 medium supplemented with 10% SVF, 1X glutamin and 1ml of gentamicin. No cell was added to the last row ("H" row) of the flat-bottomed 96-well plates in order to evaluate background  
15 absorbance of culture medium.

At the same time, two-fold serial dilutions of interferon samples were made with RPMI 1640 complete media into 96-Deep-Well plates with final concentration ranging from 6000 to 2.9 pg/ml. Interferon dilutions (50  $\mu$ l) were added to each well containing 50  $\mu$ l of Daudi cells.  
20 The total volume in each well should now be 100  $\mu$ l. Each interferon sample dilution was assessed in triplicate. Each well of the "G" row of the plates was filled with 50  $\mu$ l of RPMI 1640 complete media in order to be used as positive control. The plates are incubated for 72 hours at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere.

25 After 72 hours of growth, 20  $\mu$ l of Cell titer 96 Aqueous one solution reagent (Promega) was added to each well and incubated 1H30 at 37°C in an atmosphere of 5% CO<sub>2</sub>. To measure the amount of colored soluble formazan produced by cellular reduction of the MTS, the

-143-

absorbance of the dye was measured using an Elisa plate reader (spectramax) at 490nm.

The corrected absorbances ("H" row background value subtracted) obtained at 490nm were plotted versus concentration of cytokine. The

- 5 ED50 value was calculated by determining the X-axis value corresponding to one-half the difference between the maximum and minimum absorbance values. (ED50 = the concentration of cytokine necessary to give one-half the maximum response).

#### **D.3 Treatment of IFN $\alpha$ -2b with proteolytic preparations**

- 10 Mutants were treated with proteases in order to identify resistant molecules. The resistance of the mutant IFN  $\alpha$ -2b molecules compared to wild-type IFN  $\alpha$ -2b against enzymatic cleavage (30 min, 25 °C) by a mixture of proteases (containing 1.5 pg of each of the following proteases (1% wt/wt, Sigma):  $\alpha$ -chymotrypsin, carboxypeptidase,
- 15 endoproteinase Arg-C, endoproteinase Asp-N, endoproteinase Glu-C, endoproteinase Lys-C, and trypsin) was determined. At the end of the incubation time, 10  $\mu$ l of anti-proteases complete, mini EDTA free, Roche (one tablet was dissolved in 10 ml of DMEM and then diluted to 1/1000) was added to each reaction in order to inhibit protease activity.
- 20 samples were then used to determine residual antiviral or antiproliferation activities.

#### **D.4 Protease resistance - Kinetic analysis**

- The percent of residual IFN  $\alpha$ -2b activity over time of exposure to proteases was evaluated by a kinetic study using either (a) 15 pg of
- 25 chymotrypsin (10%wt/wt), (b) a lysate of human blood at dilution 1/100, (c) 1.5 pg of protease mixture, or (d) human serum. Incubation times were: 0 h, 0.5 h, 1 h, 4 h, 8 h, 16 h, 24 h and 48 h. Briefly, 20  $\mu$ l of each proteolytic sample (proteases, serum, blood) was added to 100  $\mu$ l of IFN  $\alpha$ -2b at 1500 pg/ml (500U/ml) and incubated for variable times, as

-144-

indicated. At the appropriate time points, 10  $\mu$ l of anti-proteases mixture, mini EDTA free, Roche (one tablet was dissolved in 10 ml of DMEM and then diluted to 1/500) was added to each well in order to stop proteolysis reactions. Biological activity assays were then performed as described for each sample in order to determine the residual activity at each time point.

#### **D.5 Performance**

The various biological activities, protease resistance and potency of each individual mutant were analyzed using a mathematical model and algorithm (NautScan™; described in French Patent No. 9915884; (published as International PCT application No. WO 01/44809 based on PCT n° PCT/FR00/03503). Data was processed using a Hill equation-based model that uses key feature indicators of the performance of each individual mutant. Mutants were ranked based on the values of their individual performance and those on the top of the ranking list were selected as leads.

#### **E. Pharmacokinetics of selected lead mutants in mice**

IFN $\alpha$  -2b mutants selected on the basis of their overall performance in vitro, were tested for pharmacokinetics in mice in order to have an indication of their half-life in blood in vivo. Mice were treated by subcutaneous (SC) injection with alicuots of each of a number of selected lead mutants. Blood was collected at increasing time points between 0.5 and 48 hs after injection. Immediately after collection, 20 ml of anti-protease solution were added to each blood sample. Serum was obtained for further analysis. Residual IFN- $\alpha$  activity in blood was determined using the tests described in the precedent sections for in vitro characterization. Wild-type IFN  $\alpha$  (that had been produced in bacteria under comparable conditions as the lead mutants) as well as a pegylated derivative of IFN  $\alpha$ , Pegasys (Roche), also were tested for pharmacokinetics in the same experiments.

-145-

**EXAMPLE 2**

This example demonstrates the 2-dimensional (2D) scanning of IFN $\alpha$ -2b for increased resistance to proteolysis. For results, see Figures 6(A)-6(N), 6(T) and 6(U).

- 5        **A) Identifying some or all possible target sites on the protein sequence that are susceptible to digestion by one or more specific proteases (these sites are the is-HITs).**

Because IFN $\alpha$ -2b is administered as a therapeutic protein in the blood stream, a set of proteases was identified that were expected to  
10 broadly mimic the protease contents in serum. From that list of proteases, a list of the corresponding target amino acids was identified (shown in parenthesis) as follows:  $\alpha$ -chymotrypsin (F, L, M, W, and Y), endoproteinase Arg-C (R), endoproteinase Asp-N (D), endoproteinase Glu-C (E), endoproteinase Lys-C (K), and trypsin (K and R) Carboxypeptidase  
15 Y, which cleaves non-specifically from the carboxy-terminal ends of proteins, was also included in the protease mixture. The distribution of the target amino acids over the protein sequence spreads over the complete length of the protein, suggesting that the protein is potentially sensitive to protease digestion all over its sequence (FIG6A). In order to  
20 restrict the number of is-HITs to a lower number of candidate positions, the 3-dimensional structure of the IFN $\alpha$ -2b molecule (PDB code 1RH2) was used to identify and select only those residues exposed on the surface, while discarding from the candidate list those which remain buried in the structure, and therefore stay less susceptible to proteolysis  
25 (FIG6B).

- 30        **B) Identifying appropriate replacing amino acids, specific for each is-HIT, such that if used to replace one or more of the original, such as native, amino acids at that specific is-HIT, they can be expected to increase the is-HIT amino acid position's resistance to digestion by protease while at the same time, maintaining or improving the requisite biological activity of the protein (these replacing amino acids are the candidate LEADS).**

-146-

To select the candidate replacing amino acids for each is-HIT position, PAM250 matrix based analysis was used (FIG7). In one embodiment, the two highest values in PAM250 matrix, corresponding to the highest occurrence of substitutions between residues ("conservative  
5 substitutions" or "accepted point mutations"), were chosen (FIG8). Whenever only a conservative substitution was available for a given high value of the PAM250, the following higher value was selected and the totality of conservative substitutions for this value was considered. The replacement of amino acids that are exposed on the surface by cysteine  
10 residues (as shown in FIG8, while replacing Y by H or I) was explicitly avoided, since this change would potentially lead to the formation of intermolecular disulfide bonds.

Thus, based on the nature of the challenging proteases, and on evolutionary considerations as well as protein structural analysis, a  
15 strategy was defined for the rational design of human IFN $\alpha$ -2b mutants having increased resistance to proteolysis which could produce therapeutic proteins having a longer half-life. By using the algorithm PROTEOL (<http://www.infobiogen.fr>), a list of residues along the IFN $\alpha$ -2b sequence was established, which can be recognized as a substrate for  
20 different enzymes present in the serum. Because the number of residues in this particular list was high, the 3-dimensional structure of IFN $\alpha$ -2b obtained from the NMR structure of IFN $\alpha$ -2a (PDB code 1ITF) was used to select only those residues exposed to the solvent. Using this approach, 42 positions were identified, which numbering is that of the mature  
25 protein (SEQ ID NO:1): L3, P4, R12, R13, M16, R22, K23, F27, L30, K31, R33, E41, K49, E58, K70, E78, K83, Y89, E96, E107, P109, L110, M111, E113, L117, R120, K121, R125, L128, K131, E132, K133, K134, Y135, P137, M148, R149, E159, L161, R162, K164, and E165. Each of these positions was replaced by amino acid residues, such that they are

-147-

defined as compatible by the substitution matrix PAM250 while at the same time the replacement amino acids do not generate new sites for proteases.

The list of performed residue substitutions as determined by

5 PAM250 analysis is as follows:

R to H, Q

E to H, Q

K to Q, T

L to V, I

10 M to I, V

P to A, S

Y to I, H.

15 C) Systematically introducing the specific replacing amino acids (candidate LEADs) at every specific is-HIT position to generate a collection containing the corresponding mutant molecules.

The individual IFN $\alpha$ -2b mutants are generated, produced and phenotypically characterized one-by-one, in addressable arrays as set forth in Example 1, such that each mutant molecule contains initially  
20 amino acid replacements at only one is-HIT site. LEAD positions were obtained in IFN $\alpha$ -2b variants after a screening for protection against proteases, and comparing protease-untreated and protease-treated variant preparations with the corresponding conditions for the wild-type IFN $\alpha$ -2b. The percent of residual (anti-viral) activity for the IFN $\alpha$ -2b E113H variant  
25 after treatment with chymotrypsin, protease mixture, blood lysate or serum was compared to the treated wild-type IFN $\alpha$ -2b. Selected IFN $\alpha$ -2b LEADs are shown in Table 2.

A top and side view of IFN $\alpha$ -2b structure in ribbon representation (obtained from NMR structure of IFN $\alpha$ -2b, PDB code 1ITF) depict residues  
30 in "space filling" defining (1) the "receptor binding region" as deduced



-148-

either by "alanine scanning" data and studies by Piehler *et al.*, *J. Biol. Chem.*, 275:40425-40433, 2000, and Roisman *et al.*, *Proc. Natl. Acad. Sci USA*, 98:13231-13236, 2001, and (2) replacing residues (LEADs) for resistance to proteolysis.

5  
 10  
 15  
 20  
 25  
 30

**Table 2**  
**Selected LEADs of IFN $\alpha$ -2b following protease resistance**

Mutant	SEQ ID No.	Proteolysis protection	IFN antiviral activity
F27V	83	Pseudo wt	Pseudo wt
R33H	86	Pseudo wt	Pseudo wt
E41Q	87	Increased	Increased
E41H	88	Pseudo wt	Increased
E58Q	89	Increased	Pseudo wt
E58H	90	Increased	Increased
E78Q	92	Increased	Increased
E78H	93	Increased	Increased
Y89H	1303	Pseudo wt	Pseudo wt
E107Q	95	Increased	Pseudo wt
E107H	96	Increased	Pseudo wt
P109A	97	Pseudo wt	Pseudo wt
L110V	98	Pseudo wt	Pseudo wt
M111V	978	Pseudo wt	Pseudo wt
E113H	101	Increased	Pseudo wt
L117V	102	Increased	Pseudo wt
L117I	103	Increased	Pseudo wt
K121Q	104	Increased	Pseudo wt
R125H	106	Increased	Increased
R125Q	107	Increased	Increased
K133Q	114	Increased	Increased
E159H	125	Increased	Pseudo wt
E159Q	124	Increased	Pseudo wt

-149-

**EXAMPLE 3****Stabilization of IFN $\alpha$ -2b by Creation of N-Glycosylation Sites**

The creation of N-glycosylation sites on the protein was a second strategy that was used to stabilize IFN $\alpha$ -2b. Natural human IFN $\alpha$ -2b contains a unique O-glycosylation site at position 129 (the numbering corresponds to the mature protein; SEQ ID NO:1), however, no N-glycosylation sites are found in this sequence. N-glycosylation sites are defined by the N-X-S or N-X-T consensus sequences. Glycosylation has been found to play a role in protein stability. For example, glycosylation has been found to increase bioavailability via higher metabolic stability and reduced clearance. In order to generate more stable IFN $\alpha$ -2b variants, the N-glycosylation consensus sequences indicated above were introduced in the IFN $\alpha$ -2b sequence by mutagenesis. Variants of IFN $\alpha$ -2b carrying new glycosylation sites were assessed as previously described.

The structure of IFN $\alpha$ -2b is characterized by a helix bundle composed of 5 helices (A, B, C, D and E) connected with each other by a series of loops (a large AB loop and three shorter BC, CD, DE loops). The helices are joined together by two disulfide bridges between residues 1/98 and 29/138 of SEQ ID NO:1. The loops are contemplated herein to represent preferential sites for glycosylation given their exposure. Therefore, N-glycosylation sites (N-X-S or N-X-T) were created in each of the loop sequences (Table 3). Selected LEADs and pseudo wild-type IFN $\alpha$ -2b mutants after screening for addition of glycosylation sites are shown in Table 4.

25

**Table 3*****In silico* HITs for addition of glycosylation sites on IFN $\alpha$ -2b**

30

Codon No.	SEQ ID No.	N-X-S	SEQ ID No.	N-X-T
c2-4		D2N/P4S		D2N/P4T
c3-5		L3N/Q5S		L3N/Q5T
c4-6		P4N/T6S		P4N/T6T

-150-

	Codon No.	SEQ ID No.	N-X-S	SEQ ID No.	N-X-T
5	c5-7	127	Q5N/H7S	128	Q5N/H7T
	c6-8	129	T6N/S8S		T6N/S8T
	c7-9		H7N/L9S		H7N/L9T
	c8-10	130	S8N/G10S	131	S8N/G10T
	c9-11		L9N/S11S		L9N/S11T
10	c10-12	132	M21N/R23S		M21N/R23T
	c22-24		R22N/I24S		R22N/I24T
	c23-25		R23N/S25S	133	R23N/S25T
	c24-26	134	I24N/L26S		I24N/L26T
	c25-27	135	S25N/F27S	136	S25N/F27T
15	c26-28	137	L26N/S28S	138	L26N/S28T
	c28-30		S28N/L30S		S28N/L30T
	c30-32	139	L30N/D32S		L30N/D32T
	c31-33		K31N/R33S		K31N/R33T
	c32-34		D32N/H34S		D32N/H34T
20	c33-35	140	R33N/D35S	141	R33N/D35T
	c34-36	142	H34N/F36S	143	H34N/F36T
	c35-37	144	D35N/G37S		D35N/G37T
	c36-38	145	F36N/F38S	146	F36N/F38T
	c37-39	147	G37N/P39S		G37N/P39T
25	c38-40	148	F38N/Q40S	149	F38N/Q40T
	c39-41	150	P39N/E41S	151	P39N/E41T
	c40-42	152	Q40N/E42S	153	Q40N/E42T
	c41-43		E41N/F43S	155	E41N/F43T
	c42-44		E42N/G44S		E42N/G44T
30	c43-45		F43N/N45S		F43N/N45T
	c44-46	156	G44N/Q46S	157	G44N/Q46T
	c45-47	158	N45N/F47S	159	N45N/F47T
	c46-48	160	Q46N/Q48S	161	Q46N/Q48T
	c47-49	162	F47N/K49S	163	F47N/K49T
	c48-50		Q48N/A50S		Q48N/A50T
	c49-51	164	K49N/E51S		K49N/E51T
	c50-52		A50N/T52S		A50N/T52T

-151-

	Codon No.	SEQ ID No.	N-X-S	SEQ ID No.	N-X-T
	c68-70		S68N/K70S		S68N/K70T
	c70-72		K70N/S72S		K70N/S72T
	c75-77	165	A75N/D77S		A75N/D77T
	c77-79		D77N/T79S		D77N/T79T
5	C100-102	166	I100N/G102S	167	I100N/G102T
	C101-103		Q101N/V103S		Q101N/V103T
	C102-104		G102N/G104S		G102N/G104T
	C103-105	168	V103N/V105S	169	V103N/V105T
	C104-106		G104N/T106S	170	G104N/T106T
10	C105-107	171	V105N/E107S		V105N/E107T
	C10--108	172	T106N/T108S	173	T106N/T108T
	C107-109	174	E107N/P109S	175	E107N/P109T
	C108-110		T108N/I110S		T108N/I110T
	C134-136		K134N/S136S	176	K134N/S136T
15	C154-156		S154N/N156S		S154N/N156T
	C155-157		T155N/L157S		T155N/L157T
	C156-158		N156N/Q158S		N156N/Q158T
	C157-159	177	L157N/E159S	178	L157N/E159T
	C158-160		Q158N/S160S	179	Q158N/S160T
20	C159-161	180	E159N/L161S	181	E159N/L161T
	C160-162		S160N/R162S		S160N/R162T
	C161-163		L161N/S163S		L161N/S163T
	C162-164		R162N/K164S		R162N/K164T
25	C163-165		S163N/E165S		S163N/E165T

Table 4

Selected LEADs and pseudo wild-type IFN $\alpha$ -2b mutants after screening for addition of glycosylation sites

	Mutant	SEQ ID No.	Proteolysis protection	IFN antiviral activity
30	Q5N/H7S	127	Increased	Pseudo wt
	Q5N/H7T	128	ND*	ND
	P39N/E41S	150	Increased	Pseudo wt
	P39N/E41T	151	Increased	Pseudo wt
	Q40N/E42S	152	Increased	Pseudo wt
35	Q40N/E42T	153	Increased	Pseudo wt
	E41N/F43S	154	Increased	Pseudo wt

-152-

	Mutant	SEQ ID No.	Proteolysis protection	IFN antiviral activity
	E41N/F43T	155	Increased	Pseudo wt
	F43N/N45S		Increased	Pseudo wt
	F43N/N45T		ND	ND
5	G44N/Q46S	156	ND	ND
	G44N/Q46T	157	Increased	Pseudo wt
	N45N/F47S	158	Increased	Pseudo wt
	N45N/F47T	159	Increased	Pseudo wt
	Q46N/Q48S	160	Increased	Pseudo wt
	Q46N/Q48T	161	ND	ND
10	F47N/K49S	162	Increased	Pseudo wt
	F47N/K49T	163	Increased	Pseudo wt
	I100N/G102S	166	Pseudo wt	Increased
	I100N/G102T	167	Pseudo wt	Increased
	V105N/E107S	171	Pseudo wt	Increased
15	V105N/E107T		Pseudo wt	Increased
	T106N/T108S	172	Pseudo wt	Increased
	T106N/T108T	173	Pseudo wt	Increased
	E107N/P109S	174	Pseudo wt	Increased
	E107N/P109T	175	Pseudo wt	Increased
20	L157N/E159S	177	Pseudo wt	Increased
	L157N/E159T	178	Pseudo wt	Increased
	E159N/L161S	180	Pseudo wt	Increased
	E159N/L161T	181	Pseudo wt	Increased

25 \*ND, not determined

#### Example 4

##### Redesign of Interferon $\alpha$ -2b Proteins

The use of the protein redesign approach provided herein permits the generation of proteins such that they maintain requisite levels and types of biological activity compared to the native protein while their underlying amino acid sequences have been significantly changed by amino acid replacement. To first identify those amino acid positions on the IFN $\alpha$ -2b protein that are involved or not involved IFN $\alpha$ -2b protein activity, such as binding activity of IFN $\alpha$ -2b to its receptor, an Ala-scan was performed on the IFN $\alpha$ -2b sequence. For this purpose, each amino acid in the IFN $\alpha$ -2b protein sequence was individually changed into Alanine. Any other amino acid, particularly another amino acid that has a neutral effect on structure, such as Gly or Ser, also can be used. Each

-153-

resulting mutant IFN $\alpha$ -2b protein was then expressed and the antiviral activity of the individual mutants was assayed. The particular amino acid positions that are sensitive to replacement by Ala, referred to herein as  
 5     HITs would in principle not be suitable targets for amino acid replacement to increase protein stability, because of their involvement in the activity of the molecule. For the Ala-scanning, the biological activity measured for the IFN $\alpha$ -2b molecules was: *i*) their capacity to inhibit virus replication when added to permissive cells previously infected with the appropriate  
 10    virus and, *ii*) their capacity to stimulate cell proliferation when added to the appropriate cells. The relative activity of each individual mutant compared to the native protein was assayed. HITS are those mutants that produce a decrease in the activity of the protein (e.g., in this example, all the mutants with activities below about 30% of the native activity).

15       In addition, to identify the HIT positions, the Alanine-scan was used to identify the amino acid residues on IFN $\alpha$ -2b that when replaced with alanine lead to a 'pseudo-wild type' activity, i.e., those that can be replaced by alanine without leading to a decrease in biological activity.

20       A collection of mutant molecules was generated and phenotypically characterized such that IFN $\alpha$ -2b proteins with amino acid sequences different from the native ones but that still elicit the same level and type of activity as the native protein were selected. HITs and pseudo wild-type amino acid positions are shown in Table 5.

25                               **Table 5**  
**HITs and pseudo wild-type positions to IFN $\alpha$ -2b redesign**

30	Mutants	SEQ ID No.	HITs (viral activity)	Pseudo wt (viral activity)
	D2A	2	Decreased	
	P4A	3		Pseudo wt
	Q5A	4		Pseudo wt
	T6A	5		Pseudo wt
	H7A	6	Decreased	

-154-

	Mutants	SEQ ID No.	HITs (viral activity)	Pseudo wt (viral activity)
5	S8A	7	Decreased	
	L9A	8		Pseudo wt
	G10A	9		Pseudo wt
	S11A	10	Decreased	
	R12A	11	Decreased	
10	R13A	12	Decreased	
	T14A	13	Decreased	
	L15A	14	Decreased	
	M16A	15	Decreased	
	L17A	16		Pseudo wt
15	Q20A	17		Pseudo wt
	R23A	18	Decreased	
	I24A	19		Pseudo wt
	S25A	20		Pseudo wt
	L26A	21	Decreased	
20	S28A	22	Decreased	
	C29A	23	Decreased	
	L30A	24	Decreased	
	K31A	25	Decreased	
	D32A	26	Decreased	
25	R33A	27	Decreased	
	D35A	28		Pseudo wt
	G37A	29		Pseudo wt
	G39A	30		Pseudo wt
	E41A	31		Pseudo wt
30	E42	32		Pseudo wt
	F43A	33	Decreased	
	N45A	34	Decreased	
	F47A	35	Decreased	
	E51A	36		Pseudo wt
	T52A	37		Pseudo wt
	I53A	38	Decreased	
	P54A	39		Pseudo wt
	V55A	40		Pseudo wt

-155-

	Mutants	SEQ ID No.	HITs (viral activity)	Pseudo wt (viral activity)
5	L56A	41		Pseudo wt
	H57A	42		Pseudo wt
	E58A	43		Pseudo wt
	M59A	44	Decreased	
	I60A	45		Pseudo wt
10	I63A	46		Pseudo wt
	F64A	47		Pseudo wt
	N65A	48		Pseudo wt
	L66A	49	Decreased	
	F67A	50	Decreased	
15	T69A	51	Decreased	
	K70A	52	Decreased	
	D71A	53	Decreased	
	S72A	54	Decreased	
	W76A	55		Pseudo wt
20	D77A	56		Pseudo wt
	E78A	57		Pseudo wt
	L81A	58		Pseudo wt
	D82A	59	Decreased	
	K83A	60	Decreased	
25	F84A	61	Decreased	
	Y85A	62		Pseudo wt
	Y89A	63		Pseudo wt
	Q90A	64		Pseudo wt
	Q91	65	Decreased	
30	N93A	66	Decreased	
	D94A	67	Decreased	
	C98A	68	Decreased	
	V99A	69	Decreased	
	Q101A	207	Decreased	
	G104A	70		Pseudo wt
	L110A	71		Pseudo wt
	S115A	72		Pseudo wt
	Y122A	73	Decreased	



-156-

Mutants	SEQ ID No.	HITs (viral activity)	Pseudo wt (viral activity)
W140A	74	Decreased	
E146A	75		Pseudo wt

**EXAMPLE 5****5 Super LEADS of Interferon  $\alpha$ -2b Protein by Additive Directional Mutagenesis**

The use of an additive directional mutagenesis approach provided a method for the assembly of multiple mutations previously present on the individual LEAD molecules in a single mutant protein thereby generating

**10** super-LEAD mutant proteins. In this method, a collection of nucleic acid molecules encoding a library of new mutant molecules is generated, tested and phenotypically characterized one-by-one in addressable arrays. Super-LEAD mutant molecules are such that each molecule contains a variable number and type of LEAD mutations

**15** Using the LEADs obtained in Example 2, six series of mutant molecules were generated with more than one mutation per molecule as shown in Table 6. Some SuperLEAD mutant molecules were phenotypically characterized and the results are shown in Table 7. As shown in the table not all SuperLEADS have improved activity compared with the

**20** original Leads; some showed decreased activity of some type.

**Table 6**

Schema of LEADs position for SuperLEADS generation

Series 1

m1 = E41H

**25** m1 + m2 = E41H + Y89H

Series 2

m1 = E58Q

m1 + m2 = E58Q + F27V

Series 3

-157-

m1 = R125H

m1+m2 = R125H + M111V

Series 4

m1 = E159H

5 m1+m2 = E159H + Y89H

Series 5

m1 = K121Q

m1+m2 = K121Q + P109A

m1+m2+m3 = K121Q + P109A + K133Q

10 Series 6

m1 = E78H

m1+m2 = E78H + R33H

m1+m2+m3 = E78H + R33H + E58H

m1+m2+m3+m4 = E78H + R33H + E58H + L110V

15

**Table 7**  
**SuperLEADs of IFN $\alpha$ -2b multiple mutants**

	Mutant	SEQ ID No.	Proteolysis protection	IFN antiviral activity
20	E41H	88	Pseudo wt	Increased
	Y89H	1303	Pseudo wt	Pseudo wt
	E41H/Y89H/ N45D**	979	Increased	Increased
	E58Q	89	Increased	Pseudo wt
	F27V	83	Pseudo wt	Pseudo wt
25	E58Q/F27V	981	Increased	Pseudo wt
	R125H	106	Increased	Increased
	M111V	978	Pseudo wt	Pseudo wt
	R125H/M111V	986	Increased	Increased
30	E159H	125		
	Y89H	1303		
	E159H/Y89H	987		
	K121Q	104	Increased	Pseudo wt
	P109A	97	Pseudo wt	Pseudo wt

-158-

5	Mutant	SEQ ID No.	Proteolysis protection	IFN antiviral activity
	K133Q	114	Increased	Increased
	K121Q/P109A	983	Increased	Pseudo wt
	K121Q/P109A / K133Q / G102R	984	Increased	Increased
	E78H	93	Increased	Increased
	R33H	86	Pseudo wt	Pseudo wt
	E58H	89	Increased	Increased
	L110V	98	Pseudo wt	Pseudo wt
10	E78H/R33H/ E58H/L110V	982	Decreased	Decreased

Four mutants with mutations to additional those selected by the rational mutagenesis were generated in the *E. coli* MutS strain and were detected by sequencing. The mutants were the following: E41Q/ D94G  
 15 SEQ.ID No. 199; L117V/ A139G SEQ.ID No. 204; E41H/ Y89H/ N45D  
 SEQ.ID No. 198; and K121Q/ P109A/ K133Q/ G102R SEQ.ID No. 204.

#### EXAMPLE 6

##### Cloning of IFN $\beta$ in pNAUT, a mammalian cell expression plasmid

The cDNA encoding IFN  $\beta$  (see, SEQ ID No. 499) was cloned into a  
 20 mammalian expression vector, prior to the generation of the selected  
 mutations (see, Figures 6(O)-6(S) and 8(A). A collected of predesigned,  
 targeted mutants was then generated such that each individual mutant  
 was created and processed individually, physically separated from each  
 other and in addressable arrays. The mammalian expression vector pSSV9  
 25 CMV 0.3 pA (see, Example 1) was engineered as follows:

The pSSV9 CMV 0.3 pA was cut by *PvuII* and religated (this step gets rid of the ITR functions), prior to the introduction of a new *EcoRI* restriction site by Quickchange mutagenesis (Stratagene). The oligonucleotides sequences used, follow:

30 EcoRI forward primer: 5'-GCCTGTATGATTTATTGGATGTTGGAATTCCTGAT-  
 GCGGTATTTTCTCCTTACG-3' (SEQ ID NO: 218)

-159-

EcoRI reverse prime: 5'-CGTAAGGAGAAAATACCGCATCAGG-  
GAATTCCAACATCCAATAAATCATACAGGC-3' (SEQ ID NO: 219)

The construct sequence was confirmed by using the following  
oligonucleotides:

5 Seq Clal forward primer: 5'-CTGATTATCAACCGGGGTACATATGATTGACATGC-  
3' (SEQ ID NO: 220)

Seq XmnI reverse primer 5'-TACGGGATAATACCGCGCCACATAGCAGAAC-3'  
(SEQ ID NO: 221).

Then, the *XmnI*-*Clal* fragment containing the newly introduced  
10 *EcoRI* site was cloned into pSSV9 CMV 0.3 pA to replace the  
corresponding wild-type fragment and produce construct pSSV9-2*EcoRI*.

The IFN  $\beta$ -cDNA was obtained from the pIFN $\beta$ 1 (ATCC) construct.  
The sequence of the IFN  $\beta$ -cDNA was confirmed by sequencing using the  
primers below:

15 Seq forward primer: 5'-CCTGATGAAGGAGGACTC-3' (SEQ ID NO:222)

Seq reverse primer: 5'-CCAAGCAGCAGATGAGTC-3' (SEQ ID NO:223).

The verified IFN  $\beta$ -encoding cDNA first was cloned into the pTOPO-  
TA vector (Invitrogen). After checking of the cDNA sequence by  
automatic DNA sequencing, the *HindIII*-*XbaI* fragment containing the IFN  
20 cDNA was subcloned into the corresponding sites of pSSV9-2*EcoRI*,  
leading to the construct pAAV-*EcoRI*-IFN $\beta$  (pNB-AAV-IFN  $\beta$ ) Finally  
the fragment Pvu II of plasmid pNB-AAV-IFN  $\beta$  was subcloned in PvuII  
site of pUC 18 leading the final construct pUC-CMVIFN $\beta$  called  
pNAUT-IFN $\beta$ .

## 25 Production and normalization of IFN $\beta$ in mammalian cells

IFN  $\beta$  was produced in CHO Chinese Hamster Ovarian cells  
(obtained from ATCC), using Dubelcco's modified Eagle's medium  
supplemented with glucose (4.5 g/L; Gibco-BRL) and fetal bovine serum  
(5 %, Hyclone). Cells were transiently transfected as follows: 0.6 x 10<sup>5</sup>  
30 cells were seeded into 6 well plates and grown for 24 h before

-160-

transfection. Confluent cells at about 70%, were supplemented with 1.0  $\mu$ g of plasmid (from the library of IFN  $\beta$  mutants) by lipofectamine plus reagent (Invitrogen) . After gently shaking, cells were incubated for 24 h with 1 ml of culture medium supplemented with 1 % of serum. IFN  $\beta$  was  
5 obtained from culture supernatants 24 h after transfection and stored in aliquots at  $-80^{\circ}\text{C}$  until use.

Preparations of IFN  $\beta$  produced from transfected cells were screened following sequential biological assays as follows. Normalization of IFN  $\beta$  concentration from culture supernatants was performed by  
10 ELISA. IFN  $\beta$  concentrations from wild type, and mutants samples were estimated by using an international reference standard provided by the NIBSC, UK.

#### Screening and in vitro characterization of IFN $\beta$ mutants

Two activities were measured directly on IFN samples: antiviral and  
15 antiproliferation activities. Dose (concentration) - response (activity) experiments for antiviral or antiproliferation activity allowed for the calculation of the 'potency' for antiviral and antiproliferation activities, respectively. Antiviral and antiproliferation activities also were measured after incubation with proteolytic samples such as specific proteases,  
20 mixtures of selected proteases, human serum or human blood.

Assessment of activity following incubation with proteolytic samples allowed to determine the residual (antiviral or antiproliferation) activity and the respective kinetics of half-life upon exposure to proteases

#### Antiviral activity - measured by Cytopathic Effects (CPE)

25 Antiviral activity of IFN  $\beta$  was determined by the capacity of the cytokine to protect Hela cells against EMC (mouse encephalomyocarditis) virus-induced cytopathic effects. The day before, Hela cells ( $2 \times 10^5$  cells/ml) were seeded in flat-bottomed 96-well plates containing 100  $\mu$ l/well of Dulbecco's MEM-Glutamax1-sodium pyruvate medium

-161-

supplemented with 5% SVF and 0.2% of gentamicin. Cells were growth at 37°C in an atmosphere of 5% CO<sub>2</sub> for 24 hours

Two-fold serial dilutions of interferon samples were made with MEM complete media into 96-Deep-Well plates with final concentration ranging  
5 from 1600 to 0.6 pg/ml. The medium was aspirated from each well and 100 µl of interferon dilutions were added to Hela cells. Each interferon sample dilution was assessed in triplicate. The two last rows of the plates were filled with 100 µl of medium without interferon dilution samples in order to serve as controls for cells with and without virus.

10 After 24 hours of growth, a 1/1000 EMC virus dilution solution was placed in each well, except for the cell control row. Plates were returned to the CO<sub>2</sub> incubator for 48 hours. Then, the medium was aspirated and the cells were stained for 1 hour with 100 µl of Blue staining solutio to determine the proportion of intact cells. Plates were  
15 washed in a distilled water bath. The cell bound dye was extracted using 100 µl of ethylene-glycol mono-ethyl-ether (Sigma). The absorbance of the dye was measured using an Elisa plate reader (Spectramax). The antiviral activity of IFN β samples (expressed as number of IU/mg of proteins) was determined as the concentration needed for 50% protection  
20 of the cells against EMC virus-induced cytopathic effects. For proteolysis experiments, each point of the kinetic was assessed at 800 and 400 pg/ml in triplicate.

#### **Anti-proliferative activity**

25 Anti-proliferative activity of IFN β was determined by assessing the capacity of the cytokine to inhibit proliferation of Daudi cells. Daudi cells (1x10<sup>4</sup> cells) were seeded in flat-bottomed 96-well plates containing 50µl/well of RPMI 1640 medium supplemented with 10% SVF, 1X glutamine and 1ml of gentamicin. No cell was added to the last row ("H"

-162-

row) of the flat-bottomed 96-well plates in order to evaluate background absorbance of culture medium.

At the same time, two-fold serial dilutions of interferon samples were made with RPMI 1640 complete media into 96-Deep-Well plates  
5 with final concentration ranging from 6000 to 2.9 pg/ml. Interferon dilutions (50 $\mu$ l) were added to each well containing 50 $\mu$ l of Daudi cells. The total volume in each well should now be 100 $\mu$ l. Each interferon sample dilution was assessed in triplicate. Each well of the "G" row of the plates was filled with 50 $\mu$ l of RPMI 1640 complete media in order to be  
10 used as positive control. The plates were incubated for 72 hours at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere.

After 72 hours of growth, 20  $\mu$ l of Cell titer 96 Aqueous one solution reagent (Promega) was added to each well and incubated 1H30 at 37°C in an atmosphere of 5% CO<sub>2</sub>. To measure the amount of colored  
15 soluble formazan produced by cellular reduction of the MTS, the absorbance of the dye was measured using an Elisa plate reader (spectramax) at 490nm.

The corrected absorbances ("H" row background value subtracted) obtained at 490nm were plotted versus concentration of cytokine. The  
20 ED50 value was calculated by determining the X-axis value corresponding to one-half the difference between the maximum and minimum absorbance values. (ED50 = the concentration of cytokine necessary to give one-half the maximum response).

#### **Treatment of IFN $\beta$ with proteolytic preparations**

25 Mutants were treated with proteases in order to identify resistant molecules. The resistance of the mutant IFN. $\beta$  molecules compared to wild-type IFN  $\beta$  against enzymatic cleavage (120 min, 25 °C) by a mixture of proteases (containing 1.5 pg of each of the following proteases (1% wt/wt, Sigma):  $\alpha$ -chymotrypsin, carboxypeptidase,

-163-

endoproteinase Arg-C, endoproteinase Asp-N, endoproteinase Glu-C, endoproteinase Lys-C, and trypsin) was determined. At the end of the incubation time, 10  $\mu$ l of anti-proteases complete, mini EDTA free, Roche (one tablet was dissolved in 10 ml of DMEM and then diluted to 1/1000) was added to each reaction in order to inhibit protease activity. Treated samples were then used to determine residual antiviral or antiproliferation activities.

#### **Protease resistance - Kinetic analysis**

The percent of residual IFN  $\beta$  activity over time of exposure to proteases was evaluated by a kinetic study using 1.5 pg of protease mixture. Incubation times were: 0 h, 0.5 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h. Briefly, 20  $\mu$ l of each proteolytic sample (proteases, serum, blood) was added to 100  $\mu$ l of IFN  $\beta$  at 400 and 800 pg/ml and incubated for variable times, as indicated. At the appropriate time points, 10  $\mu$ l of anti-proteases mixture, mini EDTA free, Roche (one tablet was dissolved in 10 ml of DMEM and then diluted to 1/500) was added to each well in order to stop proteolysis reactions. Biological activity assays were then performed as described for each sample in order to determine the residual activity at each time point.

#### **20 Performance**

The various biological activities, protease resistance and potency of each individual mutant were analyzed using a mathematical model and algorithm (NautScan<sup>TM</sup>; Fr. Patent No. 9915884; see, also published International PCT application No. WO 01/44809 based on PCT n° 25 PCT/FR00/03503). Data was processed using a Hill equation-based model that uses key feature indicators of the performance of each individual mutant. Mutants were ranked based on the values of their individual performance and those on the top of the ranking list were selected as leads.



-164-

Using the 2D-scanning and 3D-scanning methods described above in addition to the 3-dimensional structure of IFN $\beta$ , the following amino acid target positions were identified as is-HITs on IFN $\beta$ , which numbering is that of the mature protein (SEQ ID NO:196):

- 5** By 3D-scanning (see, SEQ ID Nos. 234-289, 989-1015): D by Q at position 39, D by H at position 39, D by G at position 39, E by Q at position 42, E by H at position 42, K by Q at position 45, K by T at position 45, K by S at position 45, K by H at position 45, L by V at position 47, L by I at position 47, L by T at position 47, L by Q at position 47, L by H at position 47, L by A at position 47, K by Q at position 52, K by T at position 52, K by S at position 52, K by H at position 52, F by I at position 67, F by V at position 67, R by H at position 71, R by Q at position 71, D by H at position 73, D by G at position 73, D by Q at position 73, E by Q at position 81, E by H at position 81, E by Q at position 107, E by H at position 107, K by Q at position 108, K by T at position 108, K by S at position 108, K by H at position 108, E by Q at position 109, E by H at position 109, D by Q at position 110, D by H at position 110, D by G at position 110, F by I at position 111, F by V at position 111, R by H at position 113, R by Q at position 113, L by V at position 116, L by I at position 116, L by T at position 116, L by Q at position 116, L by H at position 116, L by A at position 116, L by V at position 120, L by I at position 120, L by T at position 120, L by Q at position 120, L by H at position 120, L by A at position 120, K by Q at position 123, K by T at position 123, K by S at position 123, K by H at position 123, R by H at position 124,, R by Q at position 124, R by H at position 128, R by Q at position 128, L by V at position 130, L by I at position 130, L by T at position 130, L by Q at position 130, L by H at position 130, L by A at position 130, K by Q at position 134, K by T at position 134, K by S at position 134, K by H at

-165-

position 134, K by Q at position 136, K by T at position 136, K by S at position 136,, K by H at position 136, E by Q at position 137, E by H at position 137, Y by H at position 163, Y by I at position 163I, R by H at position 165, R by Q at position 165.

- 5           **By 2D-scanning** (see, SEQ ID Nos.1016-1302, and table above): M by V at position 1, M by I at position 1, M by T at position 1, M by Q at position 1, M by A at position 1, L by V at position 5, L by I at position 5, L by T at position 5, L by Q at position 5, L by H at position 5, L by A at position 5, F by I at position 8, F by V at position 8, L by V at
- 10 position 9, L by I at position 9, L by T at position 9, L by Q at position 9, L by H at position 9, L by A at position 9, R by H at position 11, R by Q at position 11, F by I at position 15, F by V at position 15, K by Q at position 19, K by T at position 19, K by S at position 19, K by H at position 19, W by S at position 22, W by H at position 22, N by H at
- 15 position 25, N by S at position 25, N by Q at position 25, R by H position 27, R by Q position 27, L by V at position 28, L by I at position 28, L by T at position 28, L by Q at position 28, L by H at position 28, L by A at position 28, E by Q at position 29, E by H at position 29, Y by H at position 30, Y by I at position 30, L by V at position 32, L by I at
- 20 position 32, L by T at position 32, L by Q at position 32, L by H at position 32, L by A at position 32, K by Q at position 33, K by T at position 33, K by S at position 33, K by H at position 33, R by H at position 35, R by Q at position 35, M by V at position 36, M by I at position 36, M by T at position 36, M by Q at position 36, M by A at
- 25 position 36, D by Q at position 39, D by H at position 39, D by G at position 39, E by Q at position 42, E by H at position 42, K by Q at position 45, K by T at position 45, K by S at position 45, K by H at position 45, L by V at position 47, L by I at position 47, L by T at position 47, L by, Q at position 47, L by H at position 47, L by A at

-166-

position 47, K by Q at position 52, K by T at position 52, K by S at  
position 52, K by H at position 52, F by I at position 67, F by V at  
position 67, R by H at position 71, R by Q at position 71, D by Q at  
position 73, D by H at position 73, D by G at position 73, E by Q at  
5 position 81, E by H at position 81, E by Q at position 85, E by H at  
position 85, Y by H at position 92, Y by I at position 92, K by Q at  
position 99, K by T at position 99, K by S at position 99, K by H at  
position 99, E by Q at position 103, E by H at position 103, E by Q at  
position 104, E by H at position 104, K by Q at position 105, K by T at  
10 position 105, K by S at position 105, K by H at position 105, E by Q at  
position 107, E by H at position 107, K by Q at position 108, K by T at  
position 108, K by S at position 108, K by H at position 108, E by Q at  
position 109, E by H at position 109, D by Q at position 110, D by H at  
position 110, D by G at position 110, F by I at position 111, F by V at  
15 position 111, R by H at position 113, R by Q at position 113, L by V at  
position 116, L by I at position 116, L by T at position 116, L by Q at  
position 116, L by H at position 116, L by A at position 116, L by V at  
position 120, L by I at position 120, L by T at position 120, L by Q at  
position 120, L by H at position 120, L by A at position 120, K by Q at  
20 position 123, K by T at position 123, K by S at position 123, K by H at  
position 123, R by H at position 124, R by Q at position 124, R by H at  
position 128, R by Q at position 128, L by V at position 130, L by I at  
position 130, L by T at position 130, L by Q at position 130, L by H at  
position 130, L by A at position 130, K by Q at position 134, K by T at  
25 position 134, K by S at position 134, K by H at position 134, K by Q at  
position 136, K by T at position 136, K by S at position 136, K by H at  
position 136, E by Q at position 137, E by H at position 137, Y by H at  
position 138, Y by I at position 138, R by H at position 152, R by Q at  
position 152, Y by H at position 155, Y by I at position 155, R by H at

-167-

position 159, R by Q at position 159, Y by H at position 163, Y by I at  
position 163, R by H at position 165, R by Q at position 165, M by D at  
position 1, M by E at position 1, M by K at position 1, M by N at position  
1, M by R at position 1, M by S at position 1, L by D at position 5, L by E  
5 at position 5, L by K at position 5, L by N at position 5, L by R at position  
5, L by S at position 5, L by D at position 6, L by E at position 6, L by K  
at position 6, L by N at position 6, L by R at position 6, L by S at position  
6, L by Q at position 6, L by T at position 6, F by E at position 8, F by K  
at position 8, F by R at position 8, F by D at position 8, L by D at position  
10 9, L by E at position 9, L by K at position 9, L by N at position 9, L by R  
at position 9, L by S at position 9, Q by D at position 10, Q by E at  
position 10, Q by K at position 10, Q by N at position 10, Q by R at  
position 10, Q by S at position 10, Q by T at position 10, S by D at  
position 12, S by E at position 12, S by K at position 12, S by R at  
15 position 12, S by D at position 13, S by E at position 13, S by K at  
position 13, S by R at position 13, S by N at position 13, S by Q at  
position 13, S by T at position 13, N by D at position 14, N by E at  
position 14, N by K at position 14, N by Q at position 14, N by R at  
position 14, N by S at position 14, N by T at position 14, F by D at  
20 position 15, F by E at position 15, F by K at position 15, F by R at  
position 15, Q by D at position 16, Q by E at position 16, Q by K at  
position 16, Q by N at position 16, Q by R at position 16, Q by S at  
position 16, Q by T at position 16, C by D at position 17, C by E at  
position 17, C by K at position 17, C by N at position 17, C by Q at  
25 position 17, C by R at position 17, C by S at position 17, C by T at  
position 17, L by N at position 20, L by Q at position 20, L by R at  
position 20, L by S at position 20, L by T at position 20, L by D at  
position 20, L by E at position 20, L by K at position 20, W by D at  
position 22, W by E at position 22, W by K at position 22, W by R at

-168-

position 22, Q by D at position 23, Q by E at position 23, Q by K at  
position 23, Q by R at position 23, L by D at position 24, L by E at  
position 24, L by K at position 24, L by R at position 24, W by D at  
position 79, W by E at position 79, W by K at position 79, W by R at  
5 position 79, N by D at position 80, N by E at position 80, N by K at  
position 80, N by R at position 80, T by D at position 82, T by E at  
position 82, T by K at position 82, T by R at position 82, I by D at  
position 83, I by E at position 83, I by K at position 83, I by R at position  
83, I by N at position 83, I by Q at position 83, I by S at position 83, I by  
10 T at position 83, N by D at position 86, N by E at position 86, N by K at  
position 86, N by R at position 86, N by Q at position 86, N by S at  
position 86, N by T at position 86, L by D at position 87, L by E at  
position 87, L by K at position 87, L by R at position 87, L by N at  
position 87, L by Q at position 87, L by S at position 87, L by T at  
15 position 87, A by D at position 89, A by E at position 89, A by K at  
position 89, A by R at position 89, N by D at position 90, N by E at  
position 90, N by K at position 90, N by Q at position 90, N by R at  
position 90, N by S at position 90, N by T at position 90, V by D at  
position 91, V by E at position 91, V by K at position 91, V by N at  
20 position 91, V by Q at position 91, V by R at position 91, V by S at  
position 91, V by T at position 91, Q by D at position 94, Q by E at  
position 94, Q by Q at position 94, Q by N at position 94, Q by R at  
position 94, Q by S at position 94, Q by T at position 94, I by D at  
position 95, I by E at position 95, I by K at position 95, I by N at position  
25 95, I by Q at position 95, I by R at position 95, I by S at position 95, I by  
T at position 95, H by D at position 97, H by E at position 97, H by K at  
position 97, H by N at position 97, H by Q at position 97, H by R at  
position 97, H by S at position 97, H by T at position 97, L by D at  
position 98, L by E at position 98, L by K at position 98, L by N at

-169-

position 98, L by Q at position 98, L by R at position 98, L by S at position 98, L by T at position 98, V by D at position 101, V by E at position 101, V by K at position 101, V by N at position 101, V by Q at position 101, V by R at position 101, V by S at position 101, V by T at  
5 position 101, M by C at position 1, L by C at position 6, Q by C at position 10, S by C at position 13, Q by C at position 16, L by C at position 17, V by C at position 101, L by C at position 98, H by C at position 97, Q by C at position 94, V by C at position 91, N by C at position 90.

10

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

-170-

**WHAT IS CLAIMED IS:**

1. A modified cytokine that exhibits increased resistance to proteolysis compared to the unmodified cytokine or a modified cytokine selected from the group consisting of modified cytokines comprising a  
5 sequence of amino acids set forth in any of SEQ ID Nos. 2-181, 233-1303 or a structural homolog thereof.
2. The modified cytokine of claim 1, selected from the group consisting of a member of the interferons/interleukin-10 protein family, a member of the long-chain cytokine family and a member of the short-  
10 chain cytokine family, wherein the modified cytokine is a modified interferon  $\alpha$  of any of SEQ ID Nos. 87, 89, 90, 93, 96, 101, 103, 107, 124, 979, 980, 983, 984, 985, 986 and 987 or a cytokine modified on the basis of 3-dimensional structural homology with any of SEQ ID Nos. 87, 89, 90, 93, 96, 101, 103, 107, 124, 979, 980, 983, 984, 985, 986  
15 and 987.
3. The modified cytokine of claim 1 or claim 2 selected from the group consisting of interleukin-10 (IL-10), interferon beta (IFN $\beta$ ), interferon alpha-2a (IFN $\alpha$ -2a), interferon alpha-2b (IFN $\alpha$ -2b), and interferon gamma (IFN- $\gamma$ ), granulocyte colony stimulating factor (G-CSF), leukemia  
20 inhibitory factor (LIF), human growth hormone (hGH), ciliary neurotrophic factor (CNTF), leptin, oncostatin M, interleukin-6 (IL-6) and interleukin-12 (IL-12), erythropoietin (EPO), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-13 (IL-13), Flt3 ligand and stem cell  
25 factor (SCF).
4. The modified cytokine of claim 1, that is an interferon.
5. The modified cytokine of claim 1, that is an interferon  $\alpha$ -2b (IFN $\alpha$ -2b), interferon  $\alpha$ -2a (IFN $\alpha$ -2a), interferon  $\alpha$ -2c (IFN $\alpha$ -2c), or an interferon having the sequence set forth in SEQ ID No. 232.

-171-

6. A modified cytokine of claim 4, that is IFN $\alpha$ -2b or IFN $\alpha$ -2a or IFN $\alpha$ -2C selected from the group consisting of proteins comprising one or more single amino acid replacements in SEQ ID No. 1 or 182, corresponding to the replacement of: L by V at position 3; L by I at
- 5 position 3; P by S at position 4; P by A at position 4; R by H at position 12; R by Q at position 12; R by H at position 13; R by Q at position 13; M by V at position 16; M by I at position 16; R by H at position 22; R by Q at position 22; R or K by H at position 23; R or K by Q at position 23; F by I at position 27; F by V at position 27; L by V at position 30; L by I at
- 10 position 30; K by Q at position 31; K by T at position 31; R by H at position 33; R by Q at position 33; E by Q at position 41; E by H at position 41; K by Q at position 49; K by T at position 49; E by Q at position 58; E by H at position 58; K by Q at position 70; K by T at position 70; E by Q at position 78; E by H at position 78; K by Q at
- 15 position 83; K by T at position 83; Y by H at position 89; Y by I at position 89; E by Q at position 96; E by H at position 96; E by Q at position 107; E by H at position 107; P by S at position 109; P by A at position 109; L by V at position 110; L by I at position 110; M by V at position 111; M by I at position 111; E by Q at position 113; E by H at
- 20 position 113; L by V at position 117; L by I at position 117; R by H at position 120; R by Q at position 120; K by Q at position 121; K by T at position 121; R by H at position 125; R by Q at position 125; L by V at position 128; L by I at position 128; K by Q at position 131; K by T at position 131; E by Q at position 132; E by H at position 132; K by Q at
- 25 position 133; K by T at position 133; K by Q at position 134; K by T at position 134; Y by H at position 135; Y by I at position 135; P by S at position 137; P by A at position 137; M by V at position 148; M by I at position 148; R by H at position 149; R by Q at position 149; E by Q at position 159; E by H at position 159; L by V at position 161; L by I at



-172-

position 161; R by H at position 162; R by Q at position 162; K by Q at position 164; K by T at position 164; E by Q at position 165; and E by H at position 165,

wherein residue 1 corresponds to residue 1 of the mature IFN $\alpha$ -2b  
5 or IFN $\alpha$ -2a cytokine set forth in SEQ ID NOS:1 or 182.

7. The modified cytokine of claim 6, wherein:

the protein is human;

has more resistance to proteolysis than the unmodified protein; and

the protein is selected from the group consisting of proteins

10 comprising one or more single amino acid replacements in SEQ ID NOS:1 or 182, corresponding to: F by V at position 27; R by H at position 33; E by Q at position 41; E by H at position 41; E by Q at position 58; E by H at position 58; E by Q at position 78; E by H at position 78; Y by H at position 89; E by Q at position 107; E by H at position 107; P by A at position 109; L by V at position 110; M by V at position 111; E by Q at position 113; E by H at position 113; L by V at position 117; L by I at position 117; K by Q at position 121; K by T at position 121; R by H at position 125; R by Q at position 125; K by Q at position 133; K by T at position 133; E by Q at position 159 and E by H at position 159.

20 8. A modified IFN $\alpha$ -2b or IFN $\alpha$ -2a cytokine of claim 5 selected from the group consisting of proteins comprising one or more sets of dual-amino acid replacements in SEQ ID NOS:1 or 182, corresponding to:

D by N at position 2 and P by S at position 4;

D by N at position 2 and P by T at position 4;

25 L by N at position 3 and Q by S at position 5;

L by N at position 3 and Q by T at position 5;

P by N at position 4 and T by S at position 6;

P by N at position 4 and T by T at position 6;

Q by N at position 5 and H by S at position 7;

-173-

- Q by N at position 5 and H by T at position 7;  
T by N at position 6 and S by S at position 8;  
T by N at position 6 and S by T at position 8;  
H by N at position 7 and L by S at position 9;  
5 H by N at position 7 and L by T at position 9;  
S by N at position 8 and G by S at position 10;  
S by N at position 8 and G by T at position 10;  
L by N at position 9 and S by S at position 11;  
L by N at position 9 and S by T at position 11;  
10 M by N at position 21 and K by S at position 23;  
M by N at position 21 and K by T at position 23;  
R by N at position 22 and I by S at position 24;  
R by N at position 22 and I by T at position 24;  
R or K by N at position 23 and S by S at position 25;  
15 R or K by N at position 23 and S by T at position 25;  
I by N at position 24 and L by S at position 26;  
I by N at position 24 and L by T at position 26;  
S by N at position 25 and F by S at position 27;  
S by N at position 25 and F by T at position 27;  
20 L by N at position 26 and S by S at position 28;  
L by N at position 26 and S by T at position 28;  
S by N at position 28 and L by S at position 30;  
S by N at position 28 and L by T at position 30;  
L by N at position 30 and D by S at position 32;  
25 L by N at position 30 and D by T at position 32;  
K by N at position 31 and R by S at position 33;  
K by N at position 31 and R by T at position 33;  
D by N at position 32 and H by S at position 34;  
D by N at position 32 and H by T at position 34;

-174-

R by N at position 33 and D by S at position 35;  
R by N at position 33 and D by T at position 35;  
H by N at position 34 and F by S at position 36;  
H by N at position 34 and F by T at position 36;  
5 D by N at position 35 and G by S at position 37;  
D by N at position 35 and G by T at position 37;  
F by N at position 36 and F by S at position 38;  
F by N at position 36 and F by T at position 38;  
G by N at position 37 and P by S at position 39;  
10 G by N at position 37 and P by T at position 39;  
F by N at position 38 and Q by S at position 40;  
F by N at position 38 and Q by T at position 40;  
P by N at position 39 and E by S at position 41;  
P by N at position 39 and E by T at position 41;  
15 Q by N at position 40 and E by S at position 42;  
Q by N at position 40 and E by T at position 42;  
E by N at position 41 and F by S at position 43;  
E by N at position 41 and F by T at position 43;  
E by N at position 42 and G by S at position 44;  
20 E by N at position 42 and G by T at position 44;  
F by N at position 43 and N by S at position 45;  
F by N at position 43 and N by T at position 45;  
G by N at position 44 and Q by S at position 46;  
G by N at position 44 and Q by T at position 46;  
25 N by N at position 45 and F by S at position 47;  
N by N at position 45 and F by T at position 47;  
Q by N at position 46 and Q by S at position 48;  
Q by N at position 46 and Q by T at position 48;  
F by N at position 47 and K by S at position 49;

-175-

F by N at position 47 and K by T at position 49;  
Q by N at position 48 and A by S at position 50;  
Q by N at position 48 and A by T at position 50;  
K by N at position 49 and E by S at position 51;  
5 K by N at position 49 and E by T at position 51;  
A by N at position 50 and T by S at position 52;  
A by N at position 50 and T by T at position 52;  
S by N at position 68 and K by S at position 70;  
S by N at position 68 and K by T at position 70;  
10 K by N at position 70 and S by S at position 72;  
K by N at position 70 and S by T at position 72;  
A by N at position 75 and D by S at position 77;  
A by N at position 75 and D by T at position 77;  
D by N at position 77 and T by S at position 79;  
15 D by N at position 77 and T by T at position 79;  
I by N at position 100 and G by S at position 102;  
I by N at position 100 and G by T at position 102;  
Q by N at position 101 and V by S at position 103;  
Q by N at position 101 and V by T at position 103;  
20 G by N at position 102 and G by S at position 104;  
G by N at position 102 and G by T at position 104;  
V by N at position 103 and V by S at position 105;  
V by N at position 103 and V by T at position 105;  
G by N at position 104 and T by S at position 106;  
25 G by N at position 104 and T by T at position 106;  
V by N at position 105 and E by S at position 107;  
V by N at position 105 and E by T at position 107;  
T by N at position 106 and T by S at position 108;  
T by N at position 106 and T by T at position 108;

-176-

E by N at position 107 and P by S at position 109;  
E by N at position 107 and P by T at position 109;  
T by N at position 108 and I by S at position 110;  
T by N at position 108 and I by T at position 110;  
5 K by N at position 134 and S by S at position 136;  
K by N at position 134 and S by T at position 136;  
S by N at position 154 and N by S at position 156;  
S by N at position 154 and N by T at position 156;  
T by N at position 155 and L by S at position 157;  
10 T by N at position 155 and L by T at position 157;  
N by N at position 156 and Q by S at position 158;  
N by N at position 156 and Q by T at position 158;  
L by N at position 157 and E by S at position 159;  
L by N at position 157 and E by T at position 159;  
15 Q by N at position 158 and S by S at position 160;  
Q by N at position 158 and S by T at position 160;  
E by N at position 159 and L by S at position 161;  
E by N at position 159 and L by T at position 161;  
S by N at position 160 and R by S at position 162;  
20 S by N at position 160 and R by T at position 162;  
L by N at position 161 and S by S at position 163;  
L by N at position 161 and S by T at position 163;  
R by N at position 162 and K by S at position 164;  
R by N at position 162 and K by T at position 164;  
25 S by N at position 163 and E by S at position 165; and  
S by N at position 163 and E by T at position 165,  
wherein residue 1 corresponds to residue 1 of the mature IFN $\alpha$ -2b  
or IFN $\alpha$ -2a cytokine set forth in SEQ ID NOS:1 or 182.

-177-

9. A modified IFN $\alpha$ -2b or IFN $\alpha$ -2a mutant cytokine of claim 5 selected from the group consisting of proteins comprising one or more sets of dual amino acid replacements in SEQ ID NOS:1 or 182, corresponding to:

- 5 Q by N at position 5 and H by S at position 7;  
P by N at position 39 and E by S at position 41;  
P by N at position 39 and E by T at position 41;  
Q by N at position 40 and E by S at position 42;  
Q by N at position 40 and E by T at position 42;  
10 E by N at position 41 and F by S at position 43;  
E by N at position 41 and F by T at position 43;  
F by N at position 43 and N by S at position 45;  
G by N at position 44 and Q by T at position 46;  
N by N at position 45 and F by S at position 47;  
15 N by N at position 45 and F by T at position 47;  
Q by N at position 46 and Q by S at position 48;  
F by N at position 47 and K by S at position 49;  
F by N at position 47 and K by T at position 49;  
I by N at position 100 and G by S at position 102;  
20 I by N at position 100 and G by T at position 102;  
V by N at position 105 and E by S at position 107;  
V by N at position 105 and E by T at position 107;  
T by N at position 106 and T by S at position 108;  
T by N at position 106 and T by T at position 108;  
25 E by N at position 107 and P by S at position 109;  
E by N at position 107 and P by T at position 109;  
L by N at position 157 and E by S at position 159;  
L by N at position 157 and E by T at position 159;  
E by N at position 159 and L by S at position 161; and

-178-

E by N at position 159 and L by T at position 161.

10. A modified cytokine of claim 5, further comprising one or more pseudo-wild type mutations.

11. The modified cytokine of claim 10 that is IFN $\alpha$ -2b or  
5 IFN $\alpha$ -2a.

12. A modified IFN $\alpha$ -2b or IFN $\alpha$ -2a cytokine of claim 11, comprising one or more pseudo-wild type mutations at amino acid positions of IFN $\alpha$ -2b or IFN $\alpha$ -2a corresponding to SEQ ID NOS:1 or 182, amino acid residues: 9, 10, 17, 20, 24, 25, 35, 37, 41, 52, 54, 56, 57,  
10 58, 60, 63, 64, 65, 76, 89, and 90, wherein the mutations are selected from the group consisting of one or more of insertions, deletions and replacements of the native amino acid residue(s), wherein residue 1 corresponds to residue 1 of the mature IFN $\alpha$ -2b or IFN $\alpha$ -2a protein set forth in SEQ ID NOS:1 or 182.

15 13. A modified IFN $\alpha$ -2b or IFN $\alpha$ -2a cytokine of claim 11, comprising one wherein the pseudo-wild type replacements are one or more mutations in SEQ ID No. 1 or 182 corresponding to:

P by A at position 4; Q by A at position 5,  
T by A at position 6; L by A at position 9,  
20 LG by A at position 10; L by A at position 17,  
Q by A at position 20; I by A at position 24,  
S by A at position 25; D by A at position 35,  
G by A at position 37; G by A at position 39;  
E by A at position 41; E by A at position 42  
25 E by A at position 51; T by A at position 52,  
P by A at position 54; V by A at position 55  
L by A at position 56; H by A at position 57,  
E by A at position 58; I by A at position 60,  
I by A at position 63; F by A at position 64,

-179-

N by A at position 65; W by A at position 76,  
D by A at position 77; E by A at position 78  
L by A at position 81; Y by A at position 85  
Y by A at position 89; Q by A at position 90  
5 G by A at position 104; L by A at position 110  
S by A at position 115 and E by A at position 146.

14. A modified cytokine of claim 5, comprising one or more  
pseudo-wild type mutations at amino acid positions of IFN $\alpha$ -2b, IFN $\alpha$ -2c  
or a protein having the sequence set forth in SEQ ID No. 232  
10 corresponding amino acid residues: 4, 5, 6, 9, 10, 17, 20, 24, 25, 35,  
37, 39, 41, 42, 51, 52, 54, 56, 57, 58, 60, 63, 64, 65, 76, 77, 78, 81,  
85, 89, 90, 104, 110, 115 and 146 to SEQ ID No. 1, 182 or 232,  
wherein the mutations are selected from the group consisting of one or  
more of insertions, deletions and replacements of the native amino acid  
15 residue(s), wherein residue 1 corresponds to residue 1 of the mature  
interferon set forth in SEQ ID No.1, 182 or 232.

15. The modified cytokin of claim 14, wherein the pseudowild  
type replacements are one or more mutations selected from:  
P by A at position 4; Q by A at position 5;  
20 T by A at position 6; L by A at position 9;  
LG by A at position 10; L by A at position 17;  
Q by A at position 20; I by A at position 24;  
S by A at position 25; D by A at position 35;  
G by A at position 37; G by A at position 39;  
25 E by A at position 41; E by A at position 42;  
E by A at position 51; T by A at position 52;  
P by A at position 54; V by A at position 55;  
L by A at position 56; H by A at position 57;  
E by A at position 58; I by A at position 60;



-180-

- I by A at position 63; F by A at position 64;  
N by A at position 65; W by A at position 76;  
D by A at position 77; E by A at position 78;  
L by A at position 81; Y by A at position 85;  
5 Y by A at position 89, Q by A at position 90;  
G by A at position 104; L by A at position 110;  
S by A at position 115 and E by A at position 146, wherein the  
positions correspond to SEQ ID No. 1, 182, 185 or 232.
16. A modified cytokine of any of claims 5, 9, 12, 15 that has  
10 increased antiviral activity compared to the unmodified cytokine.
17. The modified cytokine of claim 16, wherein antiviral activity  
is assessed by measuring replication by reverse transcription  
quantification PCR (RT-qPCR).
18. A modified cytokine of any of claims 5-9, 12-15 and 17 that  
15 has more antiviral activity than antiproliferative activity compared to the  
unmodified cytokine.
19. The modified cytokine of claim 18, wherein antiproliferative  
activity is assessed by measuring cell proliferation in the presence of the  
cytokine.
- 20 20. A modified cytokine of any of claims 5-9, 12-15 and 17-19  
that binds to an IFN receptor, but exhibits decreased antiviral  
activity and decreased antiproliferative activity relative to its receptor  
binding activity when compared to the unmodified cytokine.
21. A modified cytokine of claim 1, comprising two or more  
25 mutations.
22. The modified cytokine of claim 21 that is a modified IFN $\alpha$ -2b  
cytokine.

-181-

23. A modified cytokine of claim 1, wherein the cytokine comprises the sequence of amino acids set forth in any of SEQ ID Nos 2 through 181, wherein the arginine at position 23 is replaced with a lysine.

24. A modified cytokine of any claim 1 selected from the group  
5 consisting of interleukin-10 (IL-10), interferon beta (IFN $\beta$ ), interferon alpha (IFN $\alpha$ ), interferon gamma (IFN- $\gamma$ ), granulocyte colony stimulating factor (G-CSF), leukemia inhibitory factor (LIF), human growth hormone (hGH), ciliary neurotrophic factor (CNTF), leptin, oncostatin M, interleukin-6 (IL-6) and interleukin-12 (IL-12), erythropoietin (EPO), granulocyte-macrophage  
10 colony stimulating factor (GM-CSF), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-13 (IL-13), Flt3 ligand and stem cell factor (SCF).

25. A collection of the modified cytokines of any of claims 1-24.

26. A nucleic acid molecule encoding a cytokine of any of claim  
15 of claims 1-24.

27. A vector comprising a nucleic acid molecule of claim 26.

28. A eukaryotic cell, comprising the vector of claim 27.

29. A collection of nucleic acid molecules comprising a plurality of the molecules of claim 26.

20 30. A collection of nucleic acid molecules comprising a plurality of the vectors of claim 27.

31. A method for expression of a modified cytokine, comprising: introducing a nucleic acid of claim 26 into a host; and culturing the cell, under conditions and in which the modified  
25 encoded cytokines are expressed.

32. The method of claim 31, wherein the nucleic acid is introduced into a host cell.

-182-

33. The method of claim 31 or claim 32, wherein the cytokine is a modified IFN $\alpha$ -2b, IFN $\alpha$ -2a cytokine IFN $\alpha$ -2c or interferon of SEQ ID No. 232.

34. The method of claim 31 or 32, wherein the host is a  
5 eukaryotic host cell.

35. The method of claim 34, wherein the cytokine is glycosylated.

36. The method of claim 31, wherein expression is effected *in vivo*.

10 37. The method of claim 31, wherein expression is effected *in vitro*.

38. The method of claim 31, wherein expression is effected in a cell-free system.

39. A cytokine produced by the method of claims 31.

15 40. A pharmaceutical composition, comprising a cytokine of any of claims 1-24 1 in a pharmaceutically acceptable carrier.

41. A modified cytokine of any of claims 5-22 that exhibits greater resistance to proteolysis compared to the unmodified cytokine, comprising one or more amino acid replacements at one or more positions  
20 on the cytokine corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of the IFN $\alpha$ -2b or IFN $\alpha$ -2a or IFN $\alpha$ -2c or consensus IFN $\alpha$  of SEQ ID No. 232 .

42. A modified cytokine of claim 41, wherein the resistance to proteolysis is measured by mixing it with a protease *in vitro*, incubation  
25 with blood or incubation with serum.

43. A modified cytokine of any of claims 1-24 that is a structural homolog of IFN $\alpha$ -2b, comprising one or more amino acid replacements in the cytokine structural homolog at positions corresponding to the 3-dimensional-structurally-similar modified positions within the 3-D structure

-183-

of the modified IFN $\alpha$ -2b or IFN $\alpha$ -2a or IFN $\alpha$  2c or an interferon of SEQ ID No. 232.

44. A modified cytokine of claim 43, wherein the homolog has increased resistance to proteolysis compared to its unmodified cytokine  
5 counterpart, wherein the resistance to proteolysis is measured by mixture with a protease *in vitro*, incubation with blood or incubation with serum.

45. The cytokine of claim 44 that is an IFN $\alpha$  cytokine.

46. The cytokine of claim 45, selected from the group consisting of IFN $\alpha$ -2a, IFN $\alpha$ -c, IFN $\alpha$ -2c, IFN $\alpha$ -d, IFN $\alpha$ -5, IFN $\alpha$ -6, IFN $\alpha$ -4, IFN $\alpha$ -4b,  
10 IFN $\alpha$ -I, IFN $\alpha$ -J, IFN $\alpha$ -H, IFN $\alpha$ -F, IFN $\alpha$ -8, and IFN $\alpha$ -consensus cytokine.

47. A modified cytokine of claim 1 that is modified IFN $\alpha$ -2a cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO. 182 in the IFN $\alpha$ -2a corresponding to a structurally-related modified amino acid position within the 3-dimensional  
15 structure of unmodified IFN $\alpha$ -2b, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN alpha-2a.

48. The modified IFN $\alpha$ -2a of claim 47, that is human and is  
20 selected from the group consisting of cytokines comprising one or more single amino acid replacements in SEQ ID NO: 182, corresponding to amino acid positions: 41, 58, 78, 107, 117, 125, 133 and 159.

49. A modified IFN $\alpha$ -c cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO. 183 in  
25 the IFN $\alpha$ -c corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood

-184-

lysate or by incubation with serum, compared to the unmodified IFN alpha-c.

50. The modified IFN $\alpha$ -c of claim 49, that is human and is selected from the group consisting of cytokines comprising one or more  
5 single amino acid replacements in SEQ ID NO: 183, corresponding to amino acid positions: 41, 59, 79, 108, 118, 126, 134 and 160.

51. A modified IFN $\alpha$ -c, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 185 in the IFN $\alpha$ -2c corresponding to a structurally-related modified amino acid  
10 position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\alpha$ -2c.

52. The modified IFN $\alpha$ -2c cytokine of claim 51, that is human  
15 and is selected from the group consisting of cytokines comprising one or more single amino acid replacements in SEQ ID NO: 185, corresponding to amino acid positions: 27, 33, 41, 59, 79, 90, 108, 110, 111, 112, 114, 118, 122, 126, 134 and 160.

53. An IFN $\alpha$ -d cytokine, comprising one or more amino acid  
20 replacements at one or more target positions in SEQ ID NO: 186 in the IFN $\alpha$ -d corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood  
25 lysate or by incubation with serum, compared to the unmodified IFN $\alpha$ -d.

54. The IFN $\alpha$ -d modified cytokine of claim 53, that is human and is selected from the group consisting of cytokines comprising one or more single amino acid replacements in SEQ ID NO: 186, corresponding to

-185-

amino acid positions: 27, 33, 41, 59, 79, 90, 108, 110, 111, 112, 114, 118, 122, 126, 134 and 160.

55. An IFN $\alpha$ -5 cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 187 in the
- 5 IFN $\alpha$ -5 corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\alpha$ -5.
- 10 56. The IFN $\alpha$ -5 modified cytokine of claim 55, that is human and is selected from the group consisting of cytokines comprising one or more single amino acid replacements in SEQ ID NO: 187, corresponding to amino acid positions: 27, 33, 41, 59, 79, 90, 108, 110, 111, 112, 114, 118, 122, 126, 134 and 160.
- 15 57. An IFN $\alpha$ -6 cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 188 in the IFN $\alpha$ -6 corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to
- 20 proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\alpha$ -6.
58. The IFN $\alpha$ -6 modified cytokine of claim 57, that is human and is selected from the group consisting of cytokines comprising one or more single amino acid replacements in SEQ ID NO: 188, corresponding to
- 25 amino acid positions: 27, 33, 41, 59, 79, 90, 108, 110, 111, 112, 114, 118, 122, 126, 134 and 160.
59. An IFN $\alpha$ -4 cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 189 in the IFN $\alpha$ -4 corresponding to a structurally-related modified amino acid

-186-

position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\alpha$ -4.

5           60.   The IFN $\alpha$ -4 modified cytokine of claim 59, that is human and is selected from the group consisting of cytokines comprising one or more single amino acid replacements in SEQ ID NO: 189, corresponding to amino acid positions: 27, 33, 41, 59, 79, 90, 108, 110, 111, 112, 114, 118, 122, 126, 134 and 160.

10           61.   An IFN $\alpha$ -4b cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 190 in the IFN $\alpha$ -4b corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to  
15 proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\alpha$ -4b.

            62.   The IFN $\alpha$ -4b modified cytokine of claim 61, that is human and is selected from the group consisting of cytokines comprising one or more single amino acid replacements in SEQ ID NO: 190, corresponding  
20 to amino acid positions: 27, 33, 41, 59, 79, 90, 108, 110, 111, 112, 114, 118, 122, 126, 134 and 160.

            63.   An IFN $\alpha$ -I cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 191 in the IFN $\alpha$ -I corresponding to a structurally-related modified amino acid position  
25 within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\alpha$ -I.

-187-

64. The IFN $\alpha$ -I modified cytokine of claim 63, that is human and is selected from the group consisting of cytokines comprising one or more single amino acid replacements in SEQ ID NO: 191, corresponding to amino acid positions: 27, 33, 41, 59, 79, 90, 108, 110, 111, 112, 114,  
5 118, 122, 126, 134 and 160.

65. An IFN $\alpha$ -J cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 192 in the IFN $\alpha$ -J corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of  
10 claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\alpha$ -J.

66. The IFN $\alpha$ -J modified cytokine of claim 65, that is human and is selected from the group consisting of cytokines comprising one or more  
15 single amino acid replacements in SEQ ID NO: 192, corresponding to amino acid positions: 27, 33, 41, 59, 79, 90, 108, 110, 111, 112, 114, 118, 122, 126, 134 and 160.

67. An IFN $\alpha$ -H cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 193 in the  
20 IFN $\alpha$ -H corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\alpha$ -H.

25 68. The IFN $\alpha$ -H modified cytokine of claim 67, that is human and is selected from the group consisting of cytokines comprising one or more single amino acid replacements in SEQ ID NO: 193, corresponding to amino acid positions: 27, 33, 41, 59, 79, 90, 108, 110, 111, 112, 114, 118, 122, 126, 134 and 160.



-188-

69. An IFN $\alpha$ -F cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 194 in the IFN $\alpha$ -F corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of  
5 claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\alpha$ -F.

70. The IFN $\alpha$ -F modified cytokine of claim 69, that is human and is selected from the group consisting of cytokines comprising one or more  
10 single amino acid replacements in SEQ ID NO: 194, corresponding to amino acid positions: 27, 33, 41, 59, 79, 90, 108, 110, 111, 112, 114, 118, 122, 126, 134 and 160.

71. An IFN $\alpha$ -8 cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 195 in the  
15 IFN $\alpha$ -8 corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\alpha$ -8.

20 72. The IFN $\alpha$ -8 modified cytokine of claim 71, that is human and is selected from the group consisting of cytokines comprising one or more single amino acid replacements in SEQ ID NO: 195, corresponding to amino acid positions: 27, 33, 41, 59, 79, 90, 108, 110, 111, 112, 114, 118, 122, 126, 134 and 160.

25 73. An IFN $\alpha$ -consensus cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 232 in the IFN $\alpha$ -consensus cytokine corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to

-189-

greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\alpha$ -consensus.

74. The IFN $\alpha$ -consensus modified cytokine of claim 1, that is  
5 human and is selected from the group consisting of cytokines comprising one or more single amino acid replacements in SEQ ID NO: 232, corresponding to amino acid positions: 27, 33, 41, 59, 79, 90, 108, 110, 111, 112, 114, 118, 122, 126, 134 and 160.

75. A modified IFN $\beta$  cytokine, comprising one or more amino  
10 acid replacements at one or more target positions in SEQ ID NO: 196 in the IFN $\alpha$ - $\beta$  cytokine corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a  
15 with a blood lysate or by incubation with serum, compared to the unmodified IFN $\beta$ .

76. A modified IFN $\beta$  cytokine of claim 1, comprising mutations at one or more amino acid residues of IFN $\beta$  corresponding to SEQ ID NO:196 at positions corresponding to: 39, 42, 45, 47, 52, 67, 71, 73, 81, 107,  
20 108, 109, 110, 111, 113, 116, 120, 123, 124, 128, 130, 134, 136, 137, 163 and 165, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

77. The modified IFN $\beta$  cytokine of claims 75 and 76, wherein the replacements are selected from the group consisting of amino acid  
25 substitutions in SEQ ID NO:196 corresponding to: D by Q at position 39, D by H at position 39, D by G at position 39, E by Q at position 42, E by H at position 42, K by Q at position 45, K by T at position 45, K by S at position 45, K by H at position 45, L by V at position 47, L by I at position 47, L by T at position 47, L by Q at position 47, L by H at

-190-

position 47, L by A at position 47, K by Q at position 52, K by T at  
position 52, K by S at position 52, K by H at position 52, F by I at  
position 67, F by V at position 67, R by H at position 71, R by Q at  
position 71, D by H at position 73, D by G at position 73, D by Q at  
5 position 73, E by Q at position 81, E by H at position 81, E by Q at  
position 107, E by H at position 107, K by Q at position 108, K by T at  
position 108, K by S at position 108, K by H at position 108, E by Q at  
position 109, E by H at position 109, D by Q at position 110, D by H at  
position 110, D by G at position 110, F by I at position 111, F by V at  
10 position 111, R by H at position 113, R by Q at position 113, L by V at  
position 116, L by I at position 116, L by T at position 116, L by Q at  
position 116, L by H at position 116, L by A at position 116, L by V at  
position 120, L by I at position 120, L by T at position 120, L by Q at  
position 120, L by H at position 120, L by A at position 120, K by Q at  
15 position 123, K by T at position 123, K by S at position 123, K by H at  
position 123, R by H at position 124,, R by Q at position 124, R by H at  
position 128, R by Q at position 128, L by V at position 130, L by I at  
position 130, L by T at position 130, L by Q at position 130, L by H at  
position 130, L by A at position 130, K by Q at position 134, K by T at  
20 position 134, K by S at position 134, K by H at position 134, K by Q at  
position 136, K by T at position 136, K by S at position 136,, K by H at  
position 136, E by Q at position 137, E by H at position 137, Y by H at  
position 163, Y by I at position 163I, R by H at position 165, R by Q at  
position 165, wherein the first amino acid listed is substituted by the  
25 second at the position indicated.

78. A modified IFN $\beta$ -1 cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 197 in the IFN $\beta$ -1 corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines

-191-

of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\beta$ -1.

79. A modified IFN $\beta$ -1 cytokine, comprising mutations at one or  
5 more amino acid residues of IFN $\beta$ -1 corresponding to SEQ ID NO:197: 39, 42, 45, 47, 52, 67, 71, 73, 81, 107, 108, 109, 110, 111, 113, 116, 120, 123, 124, 128, 130, 134, 136, 137, 163 and 165, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

10 80. A modified IFN $\beta$ -2a cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 198 in the IFN $\beta$ -2a corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to  
15 proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\beta$ -2a.

81. A modified IFN $\beta$ -2a cytokine, comprising mutations at one or more amino acid residues of IFN $\beta$ -2a corresponding to SEQ ID NO:198: 39, 42, 45, 47, 52, 67, 71, 73, 81, 107, 108, 109, 110, 111, 113, 116,  
20 120, 123, 124, 128, 130, 134, 136, 137, 163 and 165, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

82. A modified IFN-gamma cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO:  
25 199 in the IFN-gamma corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a

-192-

protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN-gamma.

83. A modified IFN-gamma cytokine, comprising mutations at one or more amino acid residues of IFN-gamma corresponding to SEQ ID NO:199: 33, 37, 40, 41, 42, 58, 61, 64, 65 and 66, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

84. The modified IFN-gamma cytokine of claims 82 and 83, wherein the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO:199 corresponding to:

L33V	E41Q	K58Q	D65Q
L33I	E41N	K58N	D65N
K37Q	E41H	K61Q	D66Q,
K37N	E42Q	K61N	
K40Q	E42N	K64Q	
K40N	E42H	K64N	

wherein the first amino acid listed is substituted by the second at the position indicated.

85. A modified IL-10 cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 200 in the IL-10 corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IL-10.

86. A modified IL-10 cytokine, comprising mutations at one or more amino acid residues of IL-10 corresponding to SEQ ID NO:200: 49, 50, 52, 53, 54, 55, 56, 57, 59, 60, 67, 68, 71, 72, 74, 75, 78, 81, 84, 85, 86, and 88, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

-193-

87. The modified IL-10 cytokine of claims 85 and 86, wherein the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO:200 corresponding to:

	K49Q	E54N	L60V	Y72I	E81N
5	K49N	E54H	L60I	E74Q	E81H
	E50Q	D55Q	E67Q	E74N	D84Q
	E50N	D55N	E67N	E74H	D84N
	E50H	F56I	E67H	E75Q	P85S
	L52V	F56V	M68V	E75N	P85A
10	L52I	K57Q	M68I	E75H	D86Q
	L53V	K57N	F71I	P78S	D86N
	L53I	Y59H	F71V	P78A	K88Q
	E54Q	Y59I	Y72H	E81Q	K88N,

wherein the first amino acid listed is substituted by the second at the 15 position indicated.

88. A modified erythropoietin cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 201 in the erythropoietin corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\alpha$ -2b

20 modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified erythropoietin.

89. A modified erythropoietin of claim 88, comprising mutations

25 at one or more amino acid residues of erythropoietin corresponding to SEQ ID NO:201 at positions: 43, 45, 48, 49, 52, 53, 55, 72, 75, 76, 123, 129, 130, 131, 162, and 165, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

90. The modified erythropoietin cytokine of claims 88 and 89,

30 wherein the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 201 corresponding to:

-194-

	D43Q	K52Q	E72N	P122S	R131H
	D43N	K52N	E72H	P122A	R131Q
	K45Q	R53H	L75V	D123Q	R162H
	K45N	R53Q	L75I	D123N	R162Q
5	F48I	E55Q	R76H	P129S	D165Q
	F48V	E55N	R76Q	P129A	D165N
	Y49H	E55H	P121S	L130V	
	Y49I	E72Q	P121A	L130I	

wherein the first amino acid listed is substituted by the second at the  
10 position indicated.

91. A modified GM-CSF cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 202 in the GM-CSF corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of erythropoietin modified  
15 cytokines of claims 88-90, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified GM-CSF.

92. A modified GM-CSF cytokine, comprising mutations at one or  
20 more amino acid residues of GM-CSF corresponding to SEQ ID NO: 202: 38, 41, 45, 46, 48, 49, 51, 60, 63, 67, 92, 93, 119, 120, 123, and 124, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

93. The modified GM-CSF cytokine of claims 91 and 92, wherein  
25 the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 202 corresponding to:

-195-

	E38Q	D48Q	K63Q	F119V
	E38N	D48N	K63N	D120Q
	E38H	L49V	R67H	D120N
	E41Q	L49I	R67Q	E123Q
5	E41N	E51Q	P92S	E123N
	E41H	E51N	P92A	E123H
	E45Q	E51H	E93Q	P124S
	E45N	E60Q	E93N	P124A,
	E45H	E60N	E93H	
10	M46V	E60H	F119I	
	M46I			

wherein the first amino acid listed is substituted by the second at the position indicated.

94. A modified Flt3 ligand cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 203 in the Flt3 ligand corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of erythropoietin modified cytokines of claims 88-90, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified Flt3 ligand.

95. A modified Flt3 ligand cytokine, comprising mutations at one or more amino acid residues of Flt3 ligand corresponding to SEQ ID NO: 203: 3, 40, 42, 43, 55, 58, 59, 61, 89, 90, 91, 95, and 96, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

96. The modified Flt3 ligand cytokine of claims 94 and 95, wherein the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 203 corresponding to:



-196-

	D3Q	R55Q	P89A
	D3N	E58Q	P90S
	D40Q	E58N	P90A
	D40N	E58H	P91S
5	E42Q	R59H	P91A
	E42N	R59Q	R95H
	E42H	K61Q	R95Q
	L43V	K61N	F96I
	L43I	P89S	F96V,
10	R55H		

wherein the first amino acid listed is substituted by the second at the position indicated.

97. A modified IL-2 cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 204 in the IL-2 corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of erythropoietin modified cytokines of claims 88-90, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IL-2.

98. A modified IL-2 cytokine, comprising mutations at one or more amino acid residues of IL-2 corresponding to SEQ ID NO: 204: 43, 45, 48, 49, 52, 53, 60, 61, 65, 67, 68, 72, 100, 103, 104, 106, 107, 109, 110, and 132, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

99. The modified IL-2 cytokine of claims 97 and 98, wherein the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 204 corresponding to:

-197-

	K43Q	L53I	E68Q	Y107I
	K43N	E60Q	E68N	D109Q
	Y45H	E60N	E68H	D109N
	Y45I	E60H	L72V	E110Q
5	K48Q	E61Q	L72I	E110N
	K48N	E61N	E100Q	E110H
	K49Q	E61H	E100N	L132V
	K49N	P65S	E100H	L132I
	E52Q	P65A	F103I	E106Q
10	E52N	E67Q	F103V	E106N
	E52H	E67N	M104V	E106H
	L53V	E67H	M104I	Y107H,

wherein the first amino acid listed is substituted by the second at the  
15 position indicated.

100. A modified IL-3 cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 205 in the IL-3 corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of erythropoietin modified cytokines of  
20 claims 88-90, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IL-3.

101. A modified IL-3 cytokine, comprising mutations at one or more amino acid residues of IL-3 corresponding to SEQ ID NO: 205: 37,  
25 43, 46, 59, 63, 66, 96, 100, 101, and 103, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

102. The modified IL-3 cytokine of claims 100 and 101, wherein the replacements are selected from the group consisting of amino acid  
30 stitutions in SEQ ID NO:205 corresponding to:

-198-

	F37I	E59Q	P96A
	F37V	E59H	K100Q
	E43Q	R63H	K100N
	E43N	R63Q	D101Q
5	E43H	K66Q	D101N
	D46Q	K66N	D103Q
	D46N	P96S	D103N,

wherein the first amino acid listed is substituted by the second at the position indicated.

- 10 103. A modified SCF cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 206 in the SCF corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of erythropoietin modified cytokines of claims 88-90, wherein the replacements lead to greater resistance to
- 15 proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified SCF.

104. A modified SCF cytokine, comprising mutations at one or more amino acid residues of SCF corresponding to SEQ ID NO: 206: 27, 31, 34, 37, 54, 58, 61, 62, 63, 96, 98, 99, 100, 102, 103, 106, 107,
- 20 108, 109, 134, and 137, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

105. The modified SCF cytokine of claims 103 and 104, wherein the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 206 corresponding to:

25	M27V	D54Q	F63I	K100Q	E106H	E134N
	M27I	D54N	F63V	K100N	P107S	E134H
	K31Q	D58Q	K96Q	F102I	P107A	D137Q
	K31N	D58N	K96N	F102V	R108H	D137N
	P34S	D61Q	L98V	K103Q	R108Q	
30	P34A	D61N	L98I	K103N	L109V	
	D37Q	K62Q	K99Q	E106Q	L109I	
	D37N	K62N	K99N	E106N	E134Q	

- wherein the first amino acid listed is substituted by the second at the
- 35 position indicated.

-199-

106. A modified IL-4 cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 207 in the IL-4 corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of erythropoietin modified cytokines of 5 claims 88-90, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IL-4.

107. A modified IL-4 cytokine, comprising mutations at one or more amino acid residues of IL-4 corresponding to SEQ ID NO: 207: 26, 10 37, 53, 60, 61, 64, 66, 100, 102, 103, and 126, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

108. The modified IL-4 cytokine of claims 106 and 107, wherein the replacements are selected from the group consisting of amino acid 15 stitutions in SEQ ID NO: 207 corresponding to:

E26Q	E60Q	L66V	E103N
E26N	E60N	L66I	E103H
E26H	E60H	P100S	K126Q
K37Q	K61Q	P100A	K126N
20 K37N	K61N	K102Q	
R53H	R64H	K102N	
R53Q	R64Q	E103Q	

wherein the first amino acid listed is substituted by the second at the 25 position indicated.

109. A modified IL-5 cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 208 in the IL-5 corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of erythropoietin modified cytokines of 30 claims 88-90, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IL-5.

-200-

110. A modified IL-5 cytokine, comprising mutations at one or more amino acid residues of IL-5 corresponding to SEQ ID NO: 208: 32, 34, 39, 46, 47, 56, 84, 85, 88, 89, 90, 102, 110, and 111, wherein the mutations comprise insertions, deletions or replacements of the native 5 amino acid residue(s).

111. The modified IL-5 cytokine of claims 109 and 110, wherein the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 208 corresponding to:

	R32H	E47N	E88N	E110Q
10	R32Q	E47H	E88H	E110N
	P34S	E56Q	E89Q	E110H
	P34A	E56N	E89N	W111S
	K39Q	E56H	E89H	W111H,
	K39N	K84Q	R90H	
15	E46Q	K84N	R90Q	
	E46N	K85Q	E102Q	
	E46H	K85N	E102N	
	E47Q	E88Q	E102H	

20 wherein the first amino acid listed is substituted by the second at the position indicated.

112. A modified IL-13 cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 209 in the IL-13 corresponding to a structurally-related modified amino acid 25 position within the 3-dimensional structure of erythropoietin modified cytokines of claims 88-90, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IL-13.

30 113. A modified IL-13 cytokine, comprising mutations at one or more amino acid residues of IL-13 corresponding to SEQ ID NO: 209: 32, 34, 38, 48, 79, 82, 85, 86, 88, 107, 108, 110, and 111, wherein the

-201-

mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

114. The modified IL-13 cytokine of claims 112 and 113, wherein the replacements are selected from the group consisting of amino acid

5 stitutions in SEQ ID NO: 209 corresponding to:

M32V	E48H	D86N	R110H
M32I	F79I	K88Q	R110Q
W34S	F79V	K88N	F111I
W34H	L82V	R107H	F111V,
10 L38V	L82I	R107Q	
L38I	R85H	E108Q	
E48Q	R85Q	E108N	
E48N	D86Q	E108H	

15 wherein the first amino acid listed is substituted by the second at the position indicated.

115. A modified G-CSF cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 210 in the G-CSF corresponding to a structurally-related modified amino acid  
20 position within the 3-dimensional structure of IFN $\alpha$ -2b modified cytokines of claims 5-22, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified G-CSF.

116. A modified G-CSF cytokine, comprising mutations at one or  
25 more amino acid residues of G-CSF corresponding to SEQ ID NO: 210: 61, 63, 68, 72, 86, 96, 100, 101, 131, 133, 135, 147, 169, 172, and 177, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

117. The modified G-CSF cytokine of claims 115 and 116,  
30 wherein the replacements are selected from the group consisting of amino acid stitutions in SEQ ID NO: 210 corresponding to:

-202-

W61S	F86I	E101N	F147I
W61H	F86V	E101H	F147V
P63S	E96Q	P131S	R169H
P63A	E96N	P131A	R169Q
5 P68S	E96H	L133V	R172H
P68A	P100S	L133I	R172Q
L72V	P100A	P135S	P177S
L72I	E101Q	P135A	P177A,

wherein the first amino acid listed is substituted by the second at the  
10 position indicated.

118. A modified leptin cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 211 in the leptin corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of G-CSF modified cytokines  
15 of claims 115-117, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified leptin.

119. A modified leptin cytokine, comprising mutations at one or more amino acid residues of leptin corresponding to SEQ ID NO: 211: 43,  
20 49, 99, 100, 104, 105, 107, 108, 141 and 142, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

120. The modified leptin cytokine of claims 118 and 119, wherein the replacements are selected from the group consisting of amino acid  
25 stitutions in SEQ ID NO: 211 corresponding to:

P43S	P99A	E105Q	D108N
P43A	W100S	E105N	D141Q
L49V	W100H	E105H	D141N
L49I	L104V	L107V	L142V
30 P99S	L104I	L107I	L142I,
		D108Q	

wherein the first amino acid listed is substituted by the second at the  
position indicated.

-203-

121. A modified CNTF cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 212 in the CNTF corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of G-CSF modified cytokines  
5 of claims 115-117, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified CNTF.

122. A modified CNTF cytokine, comprising mutations at one or more amino acid residues of CNTF corresponding to SEQ ID NO: 212: 62,  
10 64, 66, 67, 86, 89, 92, 100, 102, 104, 131, 132, 133, 135, 136, 138, 140, 143, 148, and 151, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

123. The modified CNTF cytokine of claims 121 and 122, wherein the replacements are selected from the group consisting of amino acid  
15 stitutions in SEQ ID NO: 212 corresponding to:

	D62Q	R89Q	E131N	E138H
	D62N	E92Q	E131H	D140Q
	W64S	E92N	Y132H	D140N
	W64H	E92H	Y132I	P143S
20	E66Q	P100S	K133Q	P143A
	E66N	P100A	K133N	D148Q
	E66H	E102Q	P135S	D148N
	L67V	E102N	P135A	L151V
	L67I	E102H	R136H	L151I
25	L86V	D104Q	R136Q	
	L86I	D104N	E138Q	
	R89H	E131Q	E138N	

wherein the first amino acid listed is substituted by the second at the position indicated.

30 124. A modified LIF cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 213 in the LIF corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of G-CSF modified cytokines of claims



-204-

115-117, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified LIF.

125. A modified LIF cytokine, comprising mutations at one or  
5 more amino acid residues of LIF corresponding to SEQ ID NO: 213: 69, 70, 85, 99, 102, 104, 106, 109, 137, 143, 146, 148, 149, 153, 154, and 156, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

126. The modified LIF cytokine of claims 124 and 125, wherein  
10 the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 213 corresponding to:

P69S	K102N	D143Q	K153N
P69A	L104V	D143N	D154Q
F70I	L104I	Y146H	D154N
15 F70V	P106S	Y146I	F156I
R85H	P106A	P148S	F156V,
R85Q	L109V	P148A	
R99H	L109I	D149Q	
R99Q	Y137H	D149N	
20 K102Q	Y137I	K153Q	

wherein the first amino acid listed is substituted by the second at the position indicated.

127. A modified oncostatin M cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO:  
25 214 in the oncostatin M corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of G-CSF modified cytokines of claims 115-117, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the  
30 unmodified oncostatin M.

128. A modified oncostatin M cytokine, comprising mutations at one or more amino acid residues of oncostatin M corresponding to SEQ ID

-205-

NO: 214: 59, 60, 63, 65, 84, 87, 89, 91, 94, 97, 99, 100, 103, and 106, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

129. The modified oncostatin M cytokine of claims 127 and 128, wherein the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 214 corresponding to:

	E59Q	L65I	R91Q	R100Q
	E59N	R84H	K94Q	L103V
	E59H	R84Q	K94N	L103I
10	E60Q	D87Q	D97Q	E106Q
	E60N	D87N	D97N	E106N
	E60H	E89Q	E99Q	E106H
	R63H	E89N	E99N	
	R63Q	E89H	E99H	
15	L65V	R91H	R100H	

wherein the first amino acid listed is substituted by the second at the position indicated.

130. A modified IL-12 cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 215 in the IL-12 corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of G-CSF modified cytokines of claims 115-117, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IL-12.

131. A modified IL-12 cytokine, comprising mutations at one or more amino acid residues of IL-12 corresponding to SEQ ID NO: 215: 56, 61, 66, 67, 68, 70, 72, 75, 78, 79, 82, 89, 92, 93, 107, 110, 111, 115, 117, 124, 125, 127, 128, 129, and 189, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

-206-

132. The modified IL-12 cytokine of claims 130 and 131, wherein the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 215 corresponding to:

	K56Q	E72Q	R92H	K117Q
5	K56N	E72N	R92Q	K117N
	E61Q	E72H	K93Q	L124V
	E61N	L75V	K93N	L124I
	E61H	L75I	E107Q	M125V
	L66V	R78H	E107N	M125I
10	L66I	R78Q	E107H	P127S
	E67Q	E79Q	K110Q	P127A
	E67N	E79N	K110N	K128Q
	E67H	E79H	M111V	K128N
	L68V	F82I	M111I	R129H
15	L68I	F82V	E115Q	R129Q
	K70Q	L89V	E115N	R189H
	K70N	L89I	E115H	R189Q,

wherein the first amino acid listed is substituted by the second at the position indicated.

20 133. A modified hGH cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 216 in the hGH corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of G-CSF modified cytokines of claims 115-117, wherein the replacements lead to greater resistance to  
25 proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified hGH.

134. A modified hGH cytokine, comprising mutations at one or more amino acid residues of hGH corresponding to SEQ ID NO: 216: 56, 59, 64, 65, 66, 88, 92, 94, 101, 129, 130, 133, 134, 140, 143, 145,  
30 146, 147, 183, and 186, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

-207-

135. The modified hGH cytokine of claims 133 and 134, wherein the replacements are selected from the group consisting of amino acid substitutions in SEQ ID NO: 216 corresponding to:

	E56Q	E66Q	L101V	R134Q	D147N
5	E56N	E66N	L101I	K140Q	R183H
	E56H	E66H	E129Q	K140N	R183Q
	P59S	E88Q	E129N	Y143H	E186Q
	P59A	E88N	E129H	Y143I	E186N
	R64H	E88H	D130Q	K145Q	E186H
10	R64Q	F92I	D130N	K145N	
	E65Q	F92V	P133S	F146I	
	E65N	R94H	P133A	F146V	
	E65H	R94Q	R134H	D147Q	

wherein the first amino acid listed is substituted by the second at the  
15 position indicated.

136. A modified IL-6 cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO: 217 in the IL-6 corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of G-CSF modified cytokines of claims  
20 115-117, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IL-6.

137. A modified IL-6 cytokine, comprising mutations at one or more amino acid residues of IL-6 corresponding to SEQ ID NO: 217: 64,  
25 65, 66, 68, 69, 75, 77, 92, 98, 103, 105, 108, 133, 138, 139, 140, 149, 156, 178, and 181, wherein the mutations comprise insertions, deletions or replacements of the native amino acid residue(s).

138. The modified IL-6 cytokine of claims 136 and 137, wherein the replacements are selected from the group consisting of amino acid  
30 stitutions in SEQ ID NO: 217 corresponding to:

-208-

	P64S	F73I	R103Q	D139N
	P64A	F73V	E105Q	P140S
	K65Q	F77I	E105N	P140A
	K65N	F77V	E105H	K149Q
5	M66V	E92Q	E108Q	K149N
	M66I	E92N	E108N	W156S
	E68Q	E92H	E108H	W156H
	E68N	E98Q	D133Q	R178H
	E68H	E98N	D133N	R178Q
10	K69Q	E98H	P138S	R181H
	K69N	R103H	P138A	R181Q,
			D139Q	

wherein the first amino acid listed is substituted by the second at the position indicated.

15            139. The modified IFN $\alpha$ -2b cytokine of claims 5-22 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity to either inhibit viral replication or to stimulate cell proliferation in appropriate cells, after incubation with either mixtures of proteases, individual proteases, blood  
20 lysate or serum.

              140. The modified IFN $\alpha$ -2b cytokine of claims 5-22 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity to either inhibit viral replication in the appropriate cells or to stimulate cell proliferation of the  
25 appropriate cells, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

              141. The modified IFN $\alpha$ -2b cytokine of claims 5-22 that has increased biological activity compared to the unmodified cytokine, wherein activity is assessed by measuring the capacity to either inhibit  
30 viral replication in the appropriate cells or to stimulate cell proliferation of the appropriate cells, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

-209-

142. The modified IFN $\alpha$ -2a cytokine of claims 47 and 48 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity to either inhibit viral replication or to stimulate cell proliferation in appropriate cells, after  
5 incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

143. The modified IFN $\alpha$ -2a cytokine of claims 47 and 48 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity to either inhibit viral  
10 replication in the appropriate cells or to stimulate cell proliferation of the appropriate cells, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

144. The modified IFN $\alpha$ -2a cytokine of claims 47 and 48 that has increased biological activity compared to the unmodified cytokine,  
15 wherein activity is assessed by measuring the capacity to either inhibit viral replication in the appropriate cells or to stimulate cell proliferation of the appropriate cells, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

145. The modified IFN $\alpha$ -c cytokine of claims 49 and 50 that has  
20 increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

146. The modified IFN $\alpha$ -c cytokine of claims 49 and 50 that has decreased stability compared to the unmodified cytokine, wherein stability  
25 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

147. The modified IFN $\alpha$ -c cytokine of claims 49 and 50 that has increased biological activity compared to the unmodified cytokine, after

-210-

incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

148. The modified IFN $\alpha$ -2c cytokine of claims 51 and 52 that has increased stability compared to the unmodified cytokine, wherein stability  
5 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

149. The modified IFN $\alpha$ -2c cytokine of claims 51 and 52 that has decreased stability compared to the unmodified cytokine, wherein stability  
10 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

150. The modified IFN $\alpha$ -2c cytokine of claims 51 and 52 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

15 151. The modified IFN $\alpha$ -1d cytokine of claims 53 and 54 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

152. The modified IFN $\alpha$ -1d cytokine of claims 53 and 54 that has  
20 decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

153. The modified IFN $\alpha$ -1d cytokine of claims 53 and 54 that has increased biological activity compared to the unmodified cytokine, after  
25 incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

154. The modified IFN $\alpha$ -5 cytokine of claims 55 and 56 that has increased stability compared to the unmodified cytokine, wherein stability

-211-

is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

155. The modified IFN $\alpha$ -5 cytokine of claims 55 and 56 that has decreased stability compared to the unmodified cytokine, wherein stability  
5 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

156. The modified IFN $\alpha$ -5 cytokine of claims 55 and 56 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood  
10 lysate or serum.

157. The modified IFN $\alpha$ -6 cytokine of claims 57 and 58 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

15 158. The modified IFN $\alpha$ -6 cytokine of claims 57 and 58 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

159. The modified IFN $\alpha$ -6 cytokine of claims 57 and 58 that has  
20 increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

160. The modified IFN $\alpha$ -4 cytokine of claims 59 and 60 that has increased stability compared to the unmodified cytokine, wherein stability  
25 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

161. The modified IFN $\alpha$ -4 cytokine of claims 59 and 60 that has decreased stability compared to the unmodified cytokine, wherein stability



-212-

is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

162. The modified IFN $\alpha$ -4 cytokine of claims 59 and 60 that has increased biological activity compared to the unmodified cytokine, after 5 incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

163. The modified IFN $\alpha$ -4b cytokine of claims 61 and 62 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with 10 either mixtures of proteases, individual proteases, blood lysate or serum.

164. The modified IFN $\alpha$ -4b cytokine of claims 61 and 62 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

15 165. The modified IFN $\alpha$ -4b cytokine of claims 61 and 62 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

166. The modified IFN $\alpha$ -I cytokine of claims 63 and 64 that has 20 increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

167. The modified IFN $\alpha$ -I cytokine of claims 63 and 64 that has decreased stability compared to the unmodified cytokine, wherein stability 25 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

168. The modified IFN $\alpha$ -I cytokine of claims 63 and 64 that has increased biological activity compared to the unmodified cytokine, after

-213-

incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

169. The modified IFN $\alpha$ -J cytokine of claims 65 and 66 that has increased stability compared to the unmodified cytokine, wherein stability  
5 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

170. The modified IFN $\alpha$ -J cytokine of claims 65 and 66 that has decreased stability compared to the unmodified cytokine, wherein stability  
10 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

171. The modified IFN $\alpha$ -J cytokine of claims 65 and 66 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood  
lysate or serum.

15 172. The modified IFN $\alpha$ -H cytokine of claims 67 and 68 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

173. The modified IFN $\alpha$ -H cytokine of claims 67 and 68 that has  
20 decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

174. The modified IFN $\alpha$ -H cytokine of claims 67 and 68 that has increased biological activity compared to the unmodified cytokine, after  
25 incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

175. The modified IFN $\alpha$ -F cytokine of claims 69 and 70 that has increased stability compared to the unmodified cytokine, wherein stability

-214-

is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

176. The modified IFN $\alpha$ -F cytokine of claims 69 and 70 that has decreased stability compared to the unmodified cytokine, wherein stability  
5 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

177. The modified IFN $\alpha$ -F cytokine of claims 69 and 70 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood  
10 lysate or serum.

178. The modified IFN $\alpha$ -8 cytokine of claims 71 and 72 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

15 179. The modified IFN $\alpha$ -8 cytokine of claims 71 and 72 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

180. The modified IFN $\alpha$ -8 cytokine of claims 71 and 72 that has  
20 increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

181. The modified IFN $\alpha$  consensus cytokine of claims 73 and 74 that has increased stability compared to any of the aligned cytokines,  
25 wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

182. The modified IFN $\alpha$  consensus cytokine of claims 73 and 74 that has decreased stability compared to any of the aligned cytokines,

-215-

wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

183. The modified IFN $\alpha$  consensus cytokine of claims 73 and 74  
5 that has increased biological activity compared to any of the aligned cytokines, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

184. The modified IFN $\beta$  cytokine of claims 75-77 that has  
increased stability compared to the unmodified cytokine, wherein stability  
10 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

185. The modified IFN $\beta$  cytokine of claims 75-77 that has  
decreased stability compared to the unmodified cytokine, wherein stability  
is assessed by measuring residual biological activity after incubation with  
15 either mixtures of proteases, individual proteases, blood lysate or serum.

186. The modified IFN $\beta$  cytokine of claims 75-77 that has  
increased biological activity compared to the unmodified cytokine, after  
incubation with either mixtures of proteases, individual proteases, blood  
lysate or serum.

20 187. The modified IFN  $\beta$ -1 cytokine of claims 78 and 79 that has  
increased stability compared to the unmodified cytokine, wherein stability  
is assessed by measuring residual biological activity after incubation with  
either mixtures of proteases, individual proteases, blood lysate or serum.

188. The modified IFN  $\beta$ -1 cytokine of claims 78 and 79 that has  
25 decreased stability compared to the unmodified cytokine, wherein stability  
is assessed by measuring residual biological activity after incubation with  
either mixtures of proteases, individual proteases, blood lysate or serum.

189. The modified IFN  $\beta$ -1 cytokine of claims 78 and 79 that has  
increased biological activity compared to the unmodified cytokine, after

-216-

incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

190. The modified IFN  $\beta$ -2a cytokine of claims 80 and 81 that has increased stability compared to the unmodified cytokine, wherein stability  
5 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

191. The modified IFN  $\beta$ -2a cytokine of claims 80 and 81 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with  
10 either mixtures of proteases, individual proteases, blood lysate or serum.

192. The modified IFN  $\beta$ -2a cytokine of claims 80 and 81 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

15 193. The modified IFN-gamma cytokine of claims 82-84 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

194. The modified IFN-gamma cytokine of claims 82-84 that has  
20 decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

195. The modified IFN-gamma cytokine of claims 82-84 that has increased biological activity compared to the unmodified cytokine, after  
25 incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

196. The modified IL-10 cytokine of claims 85-87 that has increased stability compared to the unmodified cytokine, wherein stability

-217-

is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

197. The modified IL-10 cytokine of claims 85-87 that has decreased stability compared to the unmodified cytokine, wherein stability  
5 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

198. The modified IL-10 cytokine of claims 85-87 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood  
10 lysate or serum.

199. The modified erythropoietin cytokine of claims 88-90 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood  
15 lysate or serum.

200. The modified erythropoietin cytokine of claims 88-90 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood  
20 lysate or serum.

201. The modified erythropoietin cytokine of claims 88-90 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

25 202. The modified GM-CSF cytokine of claims 91-93 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

-218-

203. The modified GM-CSF cytokine of claims 91-93 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

5        204. The modified GM-CSF cytokine of claims 91-93 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

10       205. The modified Flt3 ligand cytokine of claims 94-96 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

15       206. The modified Flt3 ligand cytokine of claims 94-96 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

20       207. The modified Flt3 ligand cytokine of claims 94-96 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

208. The modified IL-2 cytokine of claims 97-99 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

25       209. The modified IL-2 cytokine of claims 97-99 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

-219-

210. The modified IL-2 cytokine of claims 97-99 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

5        211. The modified IL-3 cytokine of claims 100-102 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

10       212. The modified IL-3 cytokine of claims 100-102 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

15       213. The modified IL-3 cytokine of claims 100-102 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

20       214. The modified SCF cytokine of claims 103-105 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

25       215. The modified SCF cytokine of claims 103-105 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

216. The modified SCF cytokine of claims 103-105 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.



-220-

217. The modified IL-4 cytokine of claims 106-108 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

5        218. The modified IL-4 cytokine of claims 106-108 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

219. The modified IL-4 cytokine of claims 106-108 that has  
10 increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

220. The modified IL-5 cytokine of claims 109-111 that has increased stability compared to the unmodified cytokine, wherein stability  
15 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

221. The modified IL-5 cytokine of claims 109-111 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with  
20 either mixtures of proteases, individual proteases, blood lysate or serum.

222. The modified IL-5 cytokine of claims 109-111 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

25        223. The modified IL-13 cytokine of claims 112-114 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

-221-

224. The modified IL-13 cytokine of claims 112-114 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

5        225. The modified IL-13 cytokine of claims 112-114 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

226. The modified G-CSF cytokine of claims 115-117 that has  
10 increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

227. The modified G-CSF cytokine of claims 115-117 that has  
15 is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

228. The modified G-CSF cytokine of claims 115-117 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood  
20 lysate or serum.

229. The modified leptin cytokine of claims 118-120 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

25        230. The modified leptin cytokine of claims 118-120 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

-222-

231. The modified leptin cytokine of claims 118-120 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

5        232. The modified CNTF cytokine of claims 121-123 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

10       233. The modified CNTF cytokine of claims 121-123 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

15       234. The modified CNTF cytokine of claims 121-123 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

20       235. The modified LIF cytokine of claims 124-126 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

236. The modified LIF cytokine of claims 124-126 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

25       237. The modified LIF cytokine of claims 124-126 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

-223-

238. The modified oncostatin M cytokine of claims 127-129 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood  
5 lysate or serum.

239. The modified oncostatin M cytokine of claims 127-129 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood  
10 lysate or serum.

240. The modified oncostatin M cytokine of claims 127-129 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

15 241. The modified IL-12 cytokine of claims 130-132 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

242. The modified IL-12 cytokine of claims 130-132 that has  
20 decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

243. The modified IL-12 cytokine of claims 130-132 that has increased biological activity compared to the unmodified cytokine, after  
25 incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

244. The modified hGH of claims 133-135 that has increased stability compared to the unmodified cytokine, wherein stability is

-224-

assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

245. The modified hGH of claims 133-135 that has decreased stability compared to the unmodified cytokine, wherein stability is  
5 assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

246. The modified hGH of claims 133-135 that has increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or  
10 serum.

247. The modified IL-6 cytokine of claims 136-138 that has increased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

15 248. The modified IL-6 cytokine of claims 136-138 that has decreased stability compared to the unmodified cytokine, wherein stability is assessed by measuring residual biological activity after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

249. The modified IL-6 cytokine of claims 136-138 that has  
20 increased biological activity compared to the unmodified cytokine, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

250. A method for generating a protein or peptide molecule, having a predetermined property or activity, the method comprising:

25 (a) identifying, within a target protein or peptide or plurality thereof, one or more target amino acids, wherein:

each target amino acid is designated an *in silico*-HIT (is-HIT); and

-225-

the is-HIT target amino acids are determined by identifying structurally homologous loci between the evolving target protein and a reference protein possessing the desired activity;

(b) identifying one or more replacement amino acids, specific for  
5 each is-HIT, wherein each single amino acid replacement within the target protein or peptide is designated as a candidate LEAD protein;

(c) producing a population of sets of nucleic acid molecules that encode each of the candidate LEAD proteins, wherein each candidate LEAD protein contains a single amino acid replacement, and wherein each  
10 polynucleotide in a set encodes a candidate LEAD protein that differs by one amino acid from the target protein or peptide;

(d) introducing each set of nucleic acid molecules into host cells and expressing the encoded candidate LEAD proteins, wherein the host cells are present in an addressable array;

15 (e) individually screening the sets of encoded candidate LEAD proteins to identify one or more proteins that has an activity that differs from an activity an unmodified target protein, wherein each such protein is designated a LEAD mutant protein;

251. The method of claim 250, wherein the predetermined  
20 property or activity of the evolved modified protein is increased resistance to proteolysis.

252. The method of claim 250 or claim 251, wherein the target proteins comprise a family.

253. The method of any of claims 250-252 wherein target  
25 proteins are cytokines.

254. The method of claim 253, wherein the cytokines are selected from the group consisting of interleukin-10 (IL-10), interferon beta (IFN $\beta$ ), interferon alpha (IFN $\alpha$ ), interferon gamma (IFN- $\gamma$ ), granulocyte colony stimulating factor (G-CSF), leukemia inhibitory factor (LIF), human growth

-226-

hormone (hGH), ciliary neurotrophic factor (CNTF), leptin, oncostatin M, interleukin-6 (IL-6) and interleukin-12 (IL-12), erythropoietin (EPO), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5),  
5 interleukin-13 (IL-13), Flt3 ligand and stem cell factor (SCF).

255. The method of any of claims 250-254, wherein each candidate lead is individually prepared and screened to identify leads.

256. The method of claim 250-255, wherein the nucleic acid molecules comprise plasmids; and the cells are eukaryotic cells that are  
10 transfected with the plasmids, or the nucleic acid molecules comprise plasmids and the cells are bacterial cells.

257. The method of claim 250-256, wherein the nucleic acid molecules in step (c) are produced by site-specific mutagenesis.

258. The method of claims 250-257, further comprising:  
15 (e) generating a population of sets of nucleic acid molecules encoding a set of candidate super-LEAD proteins, wherein each candidate super-LEAD protein comprises a combination of two or more of the single amino acid mutations derived from two or more LEAD mutant proteins;  
(f) introducing each set of nucleic acid molecules encoding  
20 candidate super-LEADs into cells and expressing the encoded candidate super-LEAD proteins; and

(g) individually screening the sets of encoded candidate super-LEAD proteins to identify one or more proteins that has activity that differs from the unmodified target protein and has properties that differ from the  
25 original LEADs, wherein each such protein is designated a super-LEAD.

259. The method of claim 258, wherein the nucleic acid molecules in step (f) are generated by a method selected from among additive directional mutagenesis (ADM), multi-overlapped primer

-227-

extensions, oligonucleotide-mediated mutagenesis, nucleic acid shuffling, recombination, site-specific mutagenesis, and *de novo* synthesis.

260. The method of claim 252-259, wherein candidate leads are produced by replacing to a restricted subset of amino acids along the full 5 length of a target protein.

261. The method of claim 250-260, wherein the replacement amino acids identified in step (b) correspond to a restricted subset of the 19 remaining non-native amino acids.

262. The method of claim 250-261 wherein the nucleic acids of 10 step (c) are produced by systematically replacing each codon that is an is-HIT, with one or more codons encoding a restricted subset of the remaining amino acids, to produce nucleic acid molecules each differing by at least one codon and encoding candidate LEADs.

263. The method of claim 258, wherein the number of LEAD 15 amino acid positions generated on a single nucleic acid molecule is selected from the group consisting of: two, three, four, five, six, seven, eight, nine, ten or more LEAD amino acid positions up to all of the LEAD amino acid positions.

264. The method of claim 250-263, wherein the LEADs or super- 20 LEADs possess increased resistance to proteolysis compared to unmodified target protein.

265. The method of claim 250-264, wherein the change in activity is at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% compared to the activity of the unmodified target protein.

25 266. The method of claim 250-264, wherein the change in activity is not more than about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% compared to the activity of the unmodified target protein.



-228-

267. The method of claim 250-264, wherein the change in activity is at least about 2 times, 3 times, 4 times, 5 times, 6 times, 7 times, 8 times, 9 times, 10 times, 20 times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times, 100 times, 200 times, 5 300 times, 400 times, 500 times, 600 times, 700 times, 800 times, 900 times, 1000 times, or more greater than the activity of the unmodified target protein.

268. The method of claims 250-267, wherein the replacing amino acids are selected using Percent Accepted Mutations (PAM) matrices.

10 269. The method of claims 250-267, wherein the replacing amino acids are pseudo-wild type amino acids.

270. The method of any of claims 250-269, wherein identification of the structurally homologous loci between the evolving target protein and a reference protein possessing the desired activity, comprises:

15 (a) comparing the 3-dimensional structures of the two or more proteins to identify regions of high coincidence between their backbones, said regions designated as structurally homologous regions; and

(b) identifying is-HIT structurally homologous loci on the evolving protein that correspond to structurally related is-HIT amino acid positions  
20 within a structurally homologous region of the reference protein.

271. The method of claim 270, wherein the comparison of the 3-dimensional structures of the evolving target protein and the reference protein is based upon their 3-dimensinal strucutres not upon alignment between their respective primary sequences.

25 272. The method of claim 270 or claim 271, wherein the evolving target protein and the reference protein belong to a family of sequence-related proteins.

-229-

273. The method of any of claims 270-272, wherein the evolving target protein and the reference protein belong respectively to families of non-related proteins or sequence-non-related proteins.

274. The method of claims 250-273, wherein the degree of  
5 coincidence between the 3-dimensional structures of the evolving target protein and the reference protein is in a region selected from the group consisting of:

- (a) a small region on the two proteins;
- (b) a large region on the two proteins; and
- 10 (c) a region that covers the full length of one or both of the proteins.

275. The method of any of claims 270-274, wherein the degree of coincidence between the 3-dimensional structures of the evolving target protein and of the reference protein is determined by superposition and  
15 RMS deviation calculations using any combination of one or more of the peptide backbone atoms selected from the group consisting of: N, C, C(C=O), O and CA.

276. The method of any of claims 270-274, wherein the superposition and RMS deviation calculations are made using all of the  
20 peptide backbone atoms selected from the group consisting of: N, C, C(C=O), O and CA, when present.

277. The method of any of claims 270-276, wherein the superposition and RMS deviation calculations are carried out on a subset of regions or domains of a larger protein that adopts a structure similar to  
25 a smaller protein.

278. The method of claims 270-277, wherein the degree of coincidence between the 3-dimensional structures of the evolving target protein and the reference protein is obtained using any combination of one or more of either CATH (Class Architecture, Topology and

-230-

Homologous Superfamily); CE (Combinatorial Extension of the optimal path); FSSP (Fold Classification based of Structure-Structure Alignment of Proteins); SCOP (Structural Classification of Proteins); VAST (Vector Alignment Search Tool), and TOP.

5        279. A modified cytokine of claim 1 selected from the group consisting of modified cytokines comprising a sequence of amino acids set forth in any of SEQ ID Nos. 2-181, 233-1303 or a structural homolog thereof.

280. The modified cytokine of claim 279, selected from the group  
10 consisting of interleukin-10 (IL-10), interferon  $\alpha$ , interferon  $\beta$ , interferon  $\gamma$ , granulocyte colony stimulating factor (G-CSF), leukemia inhibitory factor (LIF), human growth hormone (hGH), ciliary neurotrophic factor (CNTF), leptin, oncostatin M, interleukin-6 (IL-6) and interleukin-12 (IL-12), erythropoietin (EPO), granulocyte-macrophage colony stimulating factor  
15 (GM-CSF), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-13 (IL-13), Flt3 ligand and stem cell factor (SCF).

281. A method of generating a modified protein or cytokine having a pre-selected altered phenotype, comprising:  
20        modifying a first protein or cytokine by a directed evolution method to produce an evolved protein or cytokine that has the altered phenotype to identify altered loci; and

      comparing the structures of one or more members of the protein or cytokine family to identify structurally homologous loci for alteration;  
25        altering the identified loci in members of the protein or cytokine family to produce proteins or cytokines that have the altered phenotype.

282. The method of claim 281, wherein directed evolution is effected by a rational directed evolution method.

-231-

283. The method of claim 281, wherein directed evolution is effected by a 2-dimensional rational scanning.

284. The method of claims 281, wherein identification of the structurally homologous loci between the evolved protein or cytokine and 5 members of the protein or cytokine family, further comprises:

(a) comparing the 3-dimensional structures of the evolved protein or cytokine with one or more members of the protein or cytokine family to identify regions of high coincidence between their backbones, said regions designated as structurally homologous regions; and

10 (b) identifying is-HIT structurally homologous loci on the members of the protein or cytokine family that correspond to structurally related is-HIT amino acid positions within a structurally homologous region of the evolved protein or cytokine.

285. The method of claim 284, wherein the comparison of the 3- 15 dimensional structures of the members of the protein or cytokine family and the evolved protein or cytokine is made irrespective of any alignment between their respective sequences.

286. The method of claim 284 or claim 285, wherein the degree of coincidence between the 3-dimensional structures of the members of 20 the protein or cytokine family and the evolved protein or cytokine is in a region selected from the group consisting of:

(a) a small region on the two proteins;

(b) a large region on the two proteins; and

(c) a region that covers the full length of one or both of the 25 proteins.

287. The method of any of claims 284-286, wherein the degree of coincidence between the 3-dimensional structures of the members of the protein or cytokine family and of the evolved protein or cytokine is determined by superposition and RMS deviation calculations using any

-232-

combination of one or more of the peptide backbone atoms selected from the group consisting of: N, C, C(C=O), O and CA.

288. The method of claim 287, wherein the superposition and RMS deviation calculations are made using all of the peptide backbone  
5 atoms present selected from group the consisting of: N, C, C(C=O), O and CA.

289. The method of claim 287 or claim 288, wherein the superposition and RMS deviation calculations are carried out on a subset of regions or domains of a larger protein that adopts a structure similar to  
10 a smaller protein.

290. The method of any of claims 284-289, wherein the degree of coincidence between the 3-dimensional structures of the members of the protein or cytokine family and the evolved protein or cytokine is obtained using any combination of one or more of either CATH (Class Architecture,  
15 Topology and Homologous Superfamily); CE (Combinatorial Extension of the optimal path); FSSP (Fold Classification based of Structure-Structure Alignment of Proteins); SCOP (Structural Classification of Proteins); VAST (Vector Alignment Search Tool), and TOP.

291. The method of claim 283, wherein the 2-dimensional rational  
20 scanning method comprises:

- (a) identifying, within the first protein or cytokine, one or more target amino acids amenable to providing the altered phenotype upon amino acid replacement, wherein each target amino acid is designated an *in silico*-HIT (is-HIT);
- 25 (b) identifying one or more replacement amino acids, specific for each is-HIT, amenable to providing the altered phenotype upon amino acid replacement, wherein each single amino acid replacement within the protein or cytokine is designated as a candidate LEAD protein;

-233-

(c) producing a population of sets of nucleic acid molecules that encode each of the candidate LEAD proteins, wherein each candidate LEAD protein comprises a single amino acid replacement, and wherein each polynucleotide in a set encodes a candidate LEAD protein that  
5 differs by one amino acid from the unmodified protein or cytokine;

(d) introducing each set of nucleic acid molecules into host cells and expressing the encoded candidate LEAD proteins, wherein the host cells are present in an addressable array;

(e) individually screening the sets of encoded candidate LEAD  
10 proteins to identify one or more candidate LEAD proteins that has activity that differs from the unmodified protein or cytokine, wherein each such protein is designated a LEAD mutant protein.

292. The method of claim 291, wherein the array comprises a solid support with wells; and each well contains one set of cells.

15 293. The method of claim 291 or claim 292, wherein the nucleic acid molecules comprise plasmids; and the cells are eukaryotic cells that are transfected with the plasmids.

294. The method of claim 291 or claim 292, wherein the nucleic acid molecules comprise plasmids and the cells are bacterial cells.

20 295. The method of any of claims 291-294, wherein the nucleic acid molecules in step (c) are produced by site-specific mutagenesis.

296. The method of any of claims 291-295, further comprising:

(f) generating a population of sets of nucleic acid molecules encoding a set of candidate super-LEAD proteins, wherein each candidate  
25 super-LEAD protein comprises a combination of two or more of the single amino acid mutations derived from two or more LEAD mutant proteins;

(g) introducing each set of nucleic acid molecules encoding candidate super-LEADs into cells and expressing the encoded candidate super-LEAD proteins; and

-234-

(h) individually screening the sets of encoded candidate super-LEAD proteins to identify one or more proteins that has activity that differs from the unmodified protein or cytokine and has properties that differ from the original LEADs, wherein each such protein is designated a super-LEAD.

5        297. The method of any of claims 296, wherein the nucleic acid molecules in step (f) are produced by a method selected from among Additive Directional Mutagenesis (ADM), multi-overlapped primer extensions, oligonucleotide-mediated mutagenesis, nucleic acid shuffling, recombination, site-specific mutagenesis, and *de novo* synthesis.

10       298. The method of any of claims 291-297, wherein the is-HITs identified in step (a) correspond to a restricted subset of amino acids along the full length target protein.

299. The method of any of claims 291-298, wherein the replacement amino acids identified in step (b) correspond to a restricted  
15 subset of the 19 remaining non-native amino acids.

300. The method of of any of claims 291-299, wherein the nucleic acids of step (c) are produced by systematically replacing each codon that is an is-HIT, with one or more codons encoding a restricted subset of the remaining amino acids, to produce nucleic acid molecules  
20 each differing by at least one codon and encoding candidate LEADs.

301. The method of claim 296, wherein the number of LEAD amino acid positions generated on a single nucleic acid molecule is selected from the group consisting of: two, three, four, five, six, seven, eight, nine, ten or more LEAD amino acid positions up to all of the LEAD  
25 amino acid positions.

302. The method of any of claims 291-301, wherein the LEADs or super-LEADs possess increased resistance to proteolysis compared to unmodified protein or cytokine.

-235-

303. The method of any of claims 291-302, wherein the change in activity is at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%, of the activity of the unmodified target protein.

304. The method of any of claims 291-302, wherein the change  
5 in activity is not more than about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%, of the activity of the unmodified target protein.

305. The method of any of claims 291-304, wherein the change in activity is at least about 2 times, 3 times, 4 times, 5 times, 6 times, 7  
10 times, 8 times, 9 times, 10 times, 20 times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times, 100 times, 200 times, 300 times, 400 times, 500 times, 600 times, 700 times, 800 times, 900 times, 1000 times, or more greater than the activity of the unmodified target protein.

15 306. A collection of the modified cytokines of any of claims 41-249.

307. A nucleic acid molecule encoding a cytokine of any of claims 41-249.

308. A vector comprising the nucleic acid molecule of claim 307.

20 309. A eukaryotic cell, comprising the vector of claim 308.

310. A collection of nucleic acid molecules comprising a plurality of the molecules of claim 307.

311. A collection of nucleic acid molecules comprising a plurality of the vectors of claim 308.

25 312. A method for expression of a modified cytokine, comprising:  
introducing a nucleic acid of claim 307 into a host; and  
culturing the cell, under conditions and in which the modified encoded cytokines are expressed.



-236-

313. The method of claim 312, wherein the nucleic acid is introduced into a host cell.

314. The method of claim 312 or 313, wherein the host is a eukaryotic host cell.

5 315. The method of claim 314, wherein the cytokine is glycosylated.

316. The method of claim 312-315, wherein expression is effected *in vivo*.

317. The method of claim 312-315, wherein expression is  
10 effected *in vitro*.

318. The method of claim 312, wherein expression is effected in a cell-free system.

319. A cytokine produced by the method of any of claims 312-318.

15 320. A modified cytokine of claim 1 that is an IFN $\alpha$ -2b, IFN $\alpha$ -2a, IFN-2c cytokine selected from the group consisting of proteins comprising one or more single amino acid replacements corresponding to the replacement of: N by D at position 45; D by G at position 94; G by R at position 102; A by G at position 139; or any combination thereof.

20 321. A modified cytokine of claim 1 that is an IFN $\alpha$ -2b, IFN $\alpha$ -2a, IFN-2c cytokine selected from selected from the group consisting of proteins comprising one or more single amino acid replacements in any of SEQ ID Nos. 1, 182, 185 or 232 or any combination thereof corresponding to the replacement: L by V at position 3; L by I at position  
25 3; P by S at position 4; P by by S at position 4; P by A at position 4; R by H at position 12; R by Q at position 12; R by H at position 13; R by Q at position 13; M by V at position 16; M by I at position 16; R by H at position 22; R by Q at position 22; R or K by H at position 23; R or K by Q at position 23; F by I at position 27; F by V at position 27; L by V at

-237-

position 30; L by I at position 30; K by Q at position 31; K by T at  
position 31; R by H at position 33; R by Q at position 33; E by Q at  
position 41; E by H at position 41; K by Q at position 49; K by T at  
position 49; E by Q at position 58; E by H at position 58; K by Q at  
5 position 70; K by T at position 70; E by Q at position 78; E by H at  
position 78; K by Q at position 83; K by T at position 83; Y by H at  
position 89; Y by I at position 89; E by Q at position 96; E by H at  
position 96; E by Q at position 107; E by H at position 107; P by S at  
position 109; P by A at position 109; L by V at position 110; L by I at  
10 position 110; M by V at position 111; M by I at position 111; E by Q at  
position 113; E by H at position 113; L by V at position 117; L by I at  
position 117; R by H at position 120; R by Q at position 120; K by Q at  
position 121; K by T at position 121; R by H at position 125; R by Q at  
position 125; L by V at position 128; L by I at position 128; K by Q at  
15 position 131; K by T at position 131; E by Q at position 132; E by H at  
position 132; K by Q at position 133; K by T at position 133; K by Q at  
position 134; K by T at position 134; Y by H at position 135; Y by I at  
position 135; P by S at position 137; P by A at position 137; M by V at  
position 148; M by I at position 148; R by H at position 149; R by Q at  
20 position 149; E by Q at position 159; E by H at position 159; L by V at  
position 161; L by I at position 161; R by H at position 162; R by Q at  
position 162; K by Q at position 164; K by T at position 164; E by Q at  
position 165; and E by H at position 165 or any combination thereof,  
wherein residue 1 corresponds to residue 1 of the mature IFN $\alpha$ -2b or  
25 IFN $\alpha$ -2a cytokine set forth in SEQ ID NOS:1 or 182.

322. A modified cytokine of claim 1 that is an IFN $\alpha$ -2b, IFN $\alpha$ -2a, IFN-2c cytokine selected from selected from the group consisting of proteins comprising one or more single amino acid replacements in any of SEQ ID Nos. 1, 182, 185 or 232 or any combination thereof corres-

-238-

ponding to the replacement L by V at position 3; L by I at position 3; P  
by S at position 4; P by A at position 4; R by H at position 12; R by Q at  
position 12; R by H at position 13; R by Q at position 13; M by V at  
position 16; M by I at position 16; R by H at position 22; R by Q at  
5 position 22; R or K by H at position 23; R or K by Q at position 23; F by I  
at position 27; F by V at position 27; L by V at position 30; L by I at  
position 30; K by Q at position 31; K by T at position 31; R by H at  
position 33; R by Q at position 33; E by Q at position 41; E by H at  
position 41; K by Q at position 49; K by T at position 49; E by Q at  
10 position 58; E by H at position 58; K by Q at position 70; K by T at  
position 70; E by Q at position 78; E by H at position 78; K by Q at  
position 83; K by T at position 83; Y by H at position 89; Y by I at  
position 89; E by Q at position 96; E by H at position 96; E by Q at  
position 107; E by H at position 107; P by S at position 109; P by A at  
15 position 109; L by V at position 110; L by I at position 110; M by V at  
position 111; M by I at position 111; E by Q at position 113; E by H at  
position 113; L by V at position 117; L by I at position 117; R by H at  
position 120; R by Q at position 120; K by Q at position 121; K by T at  
position 121; R by H at position 125; R by Q at position 125; L by V at  
20 position 128; L by I at position 128; K by Q at position 131; K by T at  
position 131; E by Q at position 132; E by H at position 132; K by Q at  
position 133; K by T at position 133; K by Q at position 134; K by T at  
position 134; Y by H at position 135; Y by I at position 135; P by S at  
position 137; P by A at position 137; M by V at position 148; M by I at  
25 position 148; R by H at position 149; R by Q at position 149; E by Q at  
position 159; E by H at position 159; L by V at position 161; L by I at  
position 161; R by H at position 162; R by Q at position 162; K by Q at  
position 164; K by T at position 164; E by Q at position 165; E by H at  
position 165; N by D at position 45; D by G at position 94; G by R at

-239-

position 102; and A by G at position 139, wherein residue 1 corresponds to residue 1 of the mature IFN $\alpha$ -2b or IFN $\alpha$ -2a cytokine set forth in SEQ ID No. 1 or 182.

323. The modified cytokine of claim 1, that is an interferon  $\beta$  5 (IFN $\beta$ ).

324. A modified IFN  $\beta$  cytokine selected from the group consisting of proteins comprising one or more single amino acid replacements in SEQ ID NOS:196, corresponding to the replacement of M by V at position 1, M by I at position 1, M by T at position 1, M by Q at position 1, M by A 10 at position 1, L by V at position 5, L by I at position 5, L by T at position 5, L by Q at position 5, L by H at position 5, L by A at position 5, F by I at position 8, F by V at position 8, L by V at position 9, L by I at position 9, L by T at position 9, L by Q at position 9, L by H at position 9, L by A at position 9, R by H at position 11, R by Q at position 11, F by 15 I at position 15, F by V at position 15, K by Q at position 19, K by T at position 19, K by S at position 19, K by H at position 19, W by S at position 22, W by H at position 22, N by H at position 25, N by S at position 25, N by Q at position 25, R by H position 27, R by Q position 27, L by V at position 28, L by I at position 28, L by T at position 28, L 20 by Q at position 28, L by H at position 28, L by A at position 28, E by Q at position 29, E by H at position 29, Y by H at position 30, Y by I at position 30, L by V at position 32, L by I at position 32, L by T at position 32, L by Q at position 32, L by H at position 32, L by A at position 32, K by Q at position 33, K by T at position 33, K by S at 25 position 33, K by H at position 33, R by H at position 35, R by Q at position 35, M by V at position 36, M by I at position 36, M by T at position 36, M by Q at position 36, M by A at position 36, D by Q at position 39, D by H at position 39, D by G at position 39, E by Q at position 42, E by H at position 42, K by Q at position 45, K by T at

-240-

position 45, K by S at position 45, K by H at position 45, L by V at  
position 47, L by I at position 47, L by T at position 47, L by, Q at  
position 47, L by H at position 47, L by A at position 47, K by Q at  
position 52, K by T at position 52, K by S at position 52, K by H at  
5 position 52, F by I at position 67, F by V at position 67, R by H at  
position 71, R by Q at position 71, D by Q at position 73, D by H at  
position 73, D by G at position 73, E by Q at position 81, E by H at  
position 81, E by Q at position 85, E by H at position 85, Y by H at  
position 92, Y by I at position 92, K by Q at position 99, K by T at  
10 position 99, K by S at position 99, K by H at position 99, E by Q at  
position 103, E by H at position 103, E by Q at position 104, E by H at  
position 104, K by Q at position 105, K by T at position 105, K by S at  
position 105, K by H at position 105, E by Q at position 107, E by H at  
position 107, K by Q at position 108, K by T at position 108, K by S at  
15 position 108, K by H at position 108, E by Q at position 109, E by H at  
position 109, D by Q at position 110, D by H at position 110, D by G at  
position 110, F by I at position 111, F by V at position 111, R by H at  
position 113, R by Q at position 113, L by V at position 116, L by I at  
position 116, L by T at position 116, L by Q at position 116, L by H at  
20 position 116, L by A at position 116, L by V at position 120, L by I at  
position 120, L by T at position 120, L by Q at position 120, L by H at  
position 120, L by A at position 120, K by Q at position 123, K by T at  
position 123, K by S at position 123, K by H at position 123, R by H at  
position 124, R by Q at position 124, R by H at position 128, R by Q at  
25 position 128, L by V at position 130, L by I at position 130, L by T at  
position 130, L by Q at position 130, L by H at position 130, L by A at  
position 130, K by Q at position 134, K by T at position 134, K by S at  
position 134, K by H at position 134, K by Q at position 136, K by T at  
position 136, K by S at position 136, K by H at position 136, E by Q at

-241-

position 137, E by H at position 137, Y by H at position 138, Y by I at  
position 138, R by H at position 152, R by Q at position 152, Y by H at  
position 155, Y by I at position 155, R by H at position 159, R by Q at  
position 159, Y by H at position 163, Y by I at position 163, R by H at  
5 position 165, R by Q at position 165, M by D at position 1, M by E at  
position 1, M by K at position 1, M by N at position 1, M by R at position  
1, M by S at position 1, L by D at position 5, L by E at position 5, L by K  
at position 5, L by N at position 5, L by R at position 5, L by S at position  
5, L by D at position 6, L by E at position 6, L by K at position 6, L by N  
10 at position 6, L by R at position 6, L by S at position 6, L by Q at position  
6, L by T at position 6, F by E at position 8, F by K at position 8, F by R  
at position 8, F by D at position 8, L by D at position 9, L by E at position  
9, L by K at position 9, L by N at position 9, L by R at position 9, L by S  
at position 9, Q by D at position 10, Q by E at position 10, Q by K at  
15 position 10, Q by N at position 10, Q by R at position 10, Q by S at  
position 10, Q by T at position 10, S by D at position 12, S by E at  
position 12, S by K at position 12, S by R at position 12, S by D at  
position 13, S by E at position 13, S by K at position 13, S by R at  
position 13, S by N at position 13, S by Q at position 13, S by T at  
20 position 13, N by D at position 14, N by E at position 14, N by K at  
position 14, N by Q at position 14, N by R at position 14, N by S at  
position 14, N by T at position 14, F by D at position 15, F by E at  
position 15, F by K at position 15, F by R at position 15, Q by D at  
position 16, Q by E at position 16, Q by K at position 16, Q by N at  
25 position 16, Q by R at position 16, Q by S at position 16, Q by T at  
position 16, C by D at position 17, C by E at position 17, C by K at  
position 17, C by N at position 17, C by Q at position 17, C by R at  
position 17, C by S at position 17, C by T at position 17, L by N at  
position 20, L by Q at position 20, L by R at position 20, L by S at

-242-

position 20, L by T at position 20, L by D at position 20, L by E at  
position 20, L by K at position 20, W by D at position 22, W by E at  
position 22, W by K at position 22, W by R at position 22, Q by D at  
position 23, Q by E at position 23, Q by K at position 23, Q by R at  
5 position 23, L by D at position 24, L by E at position 24, L by K at  
position 24, L by R at position 24, W by D at position 79, W by E at  
position 79, W by K at position 79, W by R at position 79, N by D at  
position 80, N by E at position 80, N by K at position 80, N by R at  
position 80, T by D at position 82, T by E at position 82, T by K at  
10 position 82, T by R at position 82, I by D at position 83, I by E at position  
83, I by K at position 83, I by R at position 83, I by N at position 83, I by  
Q at position 83, I by S at position 83, I by T at position 83, N by D at  
position 86, N by E at position 86, N by K at position 86, N by R at  
position 86, N by Q at position 86, N by S at position 86, N by T at  
15 position 86, L by D at position 87, L by E at position 87, L by K at  
position 87, L by R at position 87, L by N at position 87, L by Q at  
position 87, L by S at position 87, L by T at position 87, A by D at  
position 89, A by E at position 89, A by K at position 89, A by R at  
position 89, N by D at position 90, N by E at position 90, N by K at  
20 position 90, N by Q at position 90, N by R at position 90, N by S at  
position 90, N by T at position 90, V by D at position 91, V by E at  
position 91, V by K at position 91, V by N at position 91, V by Q at  
position 91, V by R at position 91, V by S at position 91, V by T at  
position 91, Q by D at position 94, Q by E at position 94, Q by Q at  
25 position 94, Q by N at position 94, Q by R at position 94, Q by S at  
position 94, Q by T at position 94, I by D at position 95, I by E at  
position 95, I by K at position 95, I by N at position 95, I by Q at position  
95, I by R at position 95, I by S at position 95, I by T at position 95, H by  
D at position 97, H by E at position 97, H by K at position 97, H by N at

-243-

position 97, H by Q at position 97, H by R at position 97, H by S at position 97, H by T at position 97, L by D at position 98, L by E at position 98, L by K at position 98, L by N at position 98, L by Q at position 98, L by R at position 98, L by S at position 98, L by T at  
5 position 98, V by D at position 101, V by E at position 101, V by K at position 101, V by N at position 101, V by Q at position 101, V by R at position 101, V by S at position 101, V by T at position 101, M by C at position 1, L by C at position 6, Q by C at position 10, S by C at position 13, Q by C at position 16, L by C at position 17, V by C at position 101,  
10 L by C at position 98, H by C at position 97, Q by C at position 94, V by C at position 91, N by C at position 90,

wherein residue 1 corresponds to residue 1 of the mature IFN $\beta$  cytokine set forth in SEQ ID NOS:196.

325. A modified cytokine of any of claims 1-4, 323 and 324,  
15 further comprising one or more pseudo-wild type mutations.

326. The modified cytokine of claim 325 that is a modified IFN $\beta$ .

327. A modified IFN $\beta$  cytokine of any of claims 323 or 324 that has increased antiviral activity compared to the unmodified cytokine.

328. The modified IFN $\beta$  cytokine of claim 327, wherein antiviral  
20 activity is assessed by measuring replication by reverse transcription quantification PCR (RT-qPCR) or CPE (cythopathic effect).

329. A modified IFN $\alpha$ -2b or IFN $\alpha$ -2a cytokine of any of claims 323, 324 or 327 that has more antiviral activity than antiproliferative activity compared to the unmodified cytokine.

25 330. The cytokine of claim 329, wherein antiproliferative activity is assessed by measuring cell proliferation in the presence of the cytokine.

331. A modified IFN $\beta$  cytokine of any of claims 323, 324, 327-330 that binds to an IFN receptor, but exhibits when compared to



-244-

unmodified IFN $\beta$ , decreased antiviral activity and decreased antiproliferative activity relative to its receptor binding activity.

332. A modified cytokine of any of claims 1-4 or 323-331 comprising two or more mutations.

5 333. The modified protein of claim 332 that is a modified IFN $\beta$  cytokine.

334. A collection of the modified cytokines of any of claims 1-4, 323-332.

335. A nucleic acid molecule encoding a cytokine of any of claims  
10 1-4, 323-332.

336. A vector comprising the nucleic acid molecule of claim 335.

337. A eukaryotic cell, comprising the vector of claim 336.

338. A collection of nucleic acid molecules comprising a plurality of the molecules of claim 337.

15 339. A collection of nucleic acid molecules comprising a plurality of the vectors of claim 338.

340. A method for expression of a modified cytokine, comprising:  
introducing a nucleic acid of claim 335 into a host; and  
culturing the cell, under conditions and in which the modified  
20 encoded cytokines are expressed.

341. The method of claim 340, wherein the nucleic acid is introduced into a host cell.

342. The method of claim 340 or 341, wherein the cytokine is a modified IFN $\beta$  cytokine.

25 343. The method of claim 340 or 341, wherein the host is a eukaryotic host cell.

344. The method of claim 343, wherein the cytokine is glycosylated.

-245-

345. The method of claim 335, wherein expression is effected *in vivo*.

346. The method of claim 345, wherein expression is effected *in vitro*.

5 347. The method of claim 345, wherein expression is effected in a cell-free system.

348. A cytokine produced by the method of any of claims 335-345.

349. A pharmaceutical composition, comprising a cytokine of any  
10 of claims 1-4, 323-333 or 348 in a pharmaceutically acceptable carrier.

350. A modified cytokine that exhibits greater resistance to proteolysis compared to the unmodified cytokine, comprising one or more amino acid replacements at one or more target positions on the cytokine corresponding to a structurally-related modified amino acid position within  
15 the 3-dimensional structure of the IFN $\beta$  modified cytokines of claims 323-333.

351. A modified cytokine of claim 350, wherein the resistance to proteolysis is measured by mixing it with a protease *in vitro*, incubation with blood or incubation with serum.

20 352. A cytokine structural homolog of an IFN $\beta$  modified cytokine of any of claims 323-333, comprising one or more amino acid replacements in the cytokine structural homolog at positions corresponding to the 3-dimensional-structurally-similar modified positions within the 3-D structure of the modified IFN $\beta$ .

25 353. A cytokine homolog of claim 352, wherein the homolog has increased resistance to proteolysis compared to its unmodified cytokine counterpart, wherein the resistance to proteolysis is measured by mixture with a protease *in vitro*, incubation with blood, or incubation with serum.

354. The cytokine of claim 353 that is an IFN $\beta$  cytokine.

-246-

355. A modified IFN $\beta$  cytokine, comprising one or more amino acid replacements at one or more target positions in SEQ ID NO. 196 in the IFN $\beta$  corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of IFN $\beta$  modified cytokines of  
5 claims 323-333, wherein the replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN $\beta$ .

356. The modified IFN $\beta$  cytokine of claims 323-333 that has increased stability compared to the unmodified cytokine, wherein stability  
10 is assessed by measuring residual biological activity to either inhibit viral replication or to stimulate cell proliferation in appropriate cells, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

357. The modified IFN $\beta$  cytokine of claims 323-333 that has decreased stability compared to the unmodified cytokine, wherein stability  
15 is assessed by measuring residual biological activity to either inhibit viral replication in the appropriate cells or to stimulate cell proliferation of the appropriate cells, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

20 358. The modified IFN $\beta$  cytokine of claims 323-333 that has increased biological activity compared to the unmodified cytokine, wherein activity is assessed by measuring the capacity to either inhibit viral replication in the appropriate cells or to stimulate cell proliferation of the appropriate cells, after incubation with either mixtures of proteases,  
25 individual proteases, blood lysate or serum.

359. A nucleic acid molecule encoding a cytokine of any of claims 350-358.

360. A vector comprising the nucleic acid molecule of claim 359.

361. A eukaryotic cell, comprising the vector of claim 360.

-247-

362. A collection of nucleic acid molecules comprising a plurality of the molecules of claim 359.

363. A collection of nucleic acid molecules comprising a plurality of the vectors of claim 360.

5        364. A method for expression of a modified cytokine, comprising:  
introducing a nucleic acid of claim 359 into a host; and  
culturing the cell, under conditions and in which the modified  
encoded cytokines are expressed.

365. The method of claim 364, wherein the nucleic acid is  
10 introduced into a host cell.

366. The method of claim 364 or 365, wherein the host is a  
eukaryotic host cell.

367. The method of claim 366, wherein the cytokine is  
glycosylated.

15        368. The method of claim 364-367, wherein expression is  
effected *in*

369. The method of claim 364-367, wherein expression is  
effected *in vitro*.

370. The method of claim 364, wherein expression is effected in a  
20 cell-free system.

371. A cytokine produced by the method of any of claims 364-  
371.

372. A modified IFN $\beta$  cytokine selected from the group consisting  
of proteins comprising one or more single amino acid replacements in SEQ  
25 ID NOS:196, or any combination thereof, corresponding to the  
replacement of:

M by V at position 1, M by I at position 1, M by T at position 1, M by Q  
at position 1, M by A at position 1, L by V at position 5, L by I at  
position 5, L by T at position 5, L by Q at position 5, L by H at position

-248-

5 , L by A at position 5 , F by I at position 8, F by V at position 8, L by V  
at position 9, L by I at position 9, L by T at position 9, L by Q at position  
9, L by H at position 9, L by A at position 9, R by H at position 11, R by  
Q at position 11, F by I at position 15 , F by V at position 15 , K by Q at  
5 position 19, K by T at position 19, K by S at position 19, K by H at  
position 19, W by S at position 22, W by H at position 22, N by H at  
position 25, N by S at position 25, N by Q at position 25, R by H  
position 27, R by Q position 27, L by V at position 28, L by I at position  
28, L by T at position 28, L by Q at position 28, L by H at position 28, L  
10 by A at position 28, E by Q at position 29, E by H at position 29, Y by H  
at position 30, Y by I at position 30, L by V at position 32, L by I at  
position 32, L by T at position 32, L by Q at position 32, L by H at  
position 32, L by A at position 32, K by Q at position 33, K by T at  
position 33, K by S at position 33, K by H at position 33, R by H at  
15 position 35, R by Q at position 35, M by V at position 36, M by I at  
position 36, M by T at position 36, M by Q at position 36, M by A at  
position 36, D by Q at position 39, D by H at position 39, D by G at  
position 39, E by Q at position 42, E by H at position 42, K by Q at  
position 45, K by T at position 45, K by S at position 45, K by H at  
20 position 45, L by V at position 47, L by I at position 47, L by T at  
position 47, L by, Q at position 47, L by H at position 47, L by A at  
position 47, K by Q at position 52, K by T at position 52, K by S at  
position 52, K by H at position 52, F by I at position 67, F by V at  
position 67, R by H at position 71, R by Q at position 71, D by Q at  
25 position 73, D by H at position 73, D by G at position 73, E by Q at  
position 81, E by H at position 81, E by Q at position 85, E by H at  
position 85, Y by H at position 92, Y by I at position 92 , K by Q at  
position 99, K by T at position 99, K by S at position 99, K by H at  
position 99, E by Q at position 103, E by H at position 103, E by Q at

-249-

position 104, E by H at position 104, K by Q at position 105, K by T at  
position 105, K by S at position 105, K by H at position 105, E by Q at  
position 107, E by H at position 107, K by Q at position 108, K by T at  
position 108, K by S at position 108, K by H at position 108, E by Q at  
5 position 109, E by H at position 109, D by Q at position 110, D by H at  
position 110, D by G at position 110, F by I at position 111, F by V at  
position 111, R by H at position 113, R by Q at position 113, L by V at  
position 116, L by I at position 116, L by T at position 116, L by Q at  
position 116, L by H at position 116, L by A at position 116, L by V at  
10 position 120, L by I at position 120, L by T at position 120, L by Q at  
position 120, L by H at position 120, L by A at position 120, K by Q at  
position 123, K by T at position 123, K by S at position 123, K by H at  
position 123, R by H at position 124, R by Q at position 124, R by H at  
position 128, R by Q at position 128, L by V at position 130, L by I at  
15 position 130, L by T at position 130, L by Q at position 130, L by H at  
position 130, L by A at position 130, K by Q at position 134, K by T at  
position 134, K by S at position 134, K by H at position 134, K by Q at  
position 136, K by T at position 136, K by S at position 136, K by H at  
position 136, E by Q at position 137, E by H at position 137, Y by H at  
20 position 138, Y by I at position 138, R by H at position 152, R by Q at  
position 152, Y by H at position 155, Y by I at position 155, R by H at  
position 159, R by Q at position 159, Y by H at position 163, Y by I at  
position 163, R by H at position 165, R by Q at position 165, M by D at  
position 1, M by E at position 1, M by K at position 1, M by N at position  
25 1, M by R at position 1, M by S at position 1, L by D at position 5, L by E  
at position 5, L by K at position 5, L by N at position 5, L by R at position  
5, L by S at position 5, L by D at position 6, L by E at position 6, L by K  
at position 6, L by N at position 6, L by R at position 6, L by S at position  
6, L by Q at position 6, L by T at position 6, F by E at position 8, F by K

-250-

at position 8, F by R at position 8, F by D at position 8, L by D at position  
9, L by E at position 9, L by K at position 9, L by N at position 9, L by R  
at position 9, L by S at position 9, Q by D at position 10 , Q by E at  
position 10 , Q by K at position 10 , Q by N at position 10 , Q by R at  
5 position 10 , Q by S at position 10 , Q by T at position 10 , S by D at  
position 12, S by E at position 12, S by K at position 12, S by R at  
position 12, S by D at position 13, S by E at position 13, S by K at  
position 13, S by R at position 13, S by N at position 13, S by Q at  
position 13, S by T at position 13, N by D at position 14, N by E at  
10 position 14, N by K at position 14, N by Q at position 14, N by R at  
position 14, N by S at position 14, N by T at position 14, F by D at  
position 15, F by E at position 15, F by K at position 15, F by R at  
position 15, Q by D at position 16, Q by E at position 16 , Q by K at  
position 16 , Q by N at position 16 , Q by R at position 16 , Q by S at  
15 position 16 , Q by T at position 16 , C by D at position 17, C by E at  
position 17, C by K at position 17, C by N at position 17, C by Q at  
position 17, C by R at position 17, C by S at position 17, C by T at  
position 17, L by N at position 20, L by Q at position 20, L by R at  
position 20, L by S at position 20, L by T at position 20, L by D at  
20 position 20, L by E at position 20, L by K at position 20, W by D at  
position 22, W by E at position 22, W by K at position 22, W by R at  
position 22, Q by D at position 23, Q by E at position 23, Q by K at  
position 23, Q by R at position 23, L by D at position 24, L by E at  
position 24, L by K at position 24, L by R at position 24, W by D at  
25 position 79, W by E at position 79, W by K at position 79, W by R at  
position 79, N by D at position 80, N by E at position 80, N by K at  
position 80, N by R at position 80, T by D at position 82, T by E at  
position 82, T by K at position 82, T by R at position 82, I by D at  
position 83, I by E at position 83, I by K at position 83, I by R at position

-251-

83, I by N at position 83, I by Q at position 83, I by S at position 83, I by  
T at position 83, N by D at position 86, N by E at position 86, N by K at  
position 86, N by R at position 86, N by Q at position 86, N by S at  
position 86, N by T at position 86, L by D at position 87, L by E at  
5 position 87, L by K at position 87, L by R at position 87, L by N at  
position 87, L by Q at position 87, L by S at position 87, L by T at  
position 87, A by D at position 89, A by E at position 89, A by K at  
position 89, A by R at position 89, N by D at position 90, N by E at  
position 90, N by K at position 90, N by Q at position 90, N by R at  
10 position 90, N by S at position 90, N by T at position 90, V by D at  
position 91, V by E at position 91, V by K at position 91, V by N at  
position 91, V by Q at position 91, V by R at position 91, V by S at  
position 91, V by T at position 91, Q by D at position 94, Q by E at  
position 94, Q by Q at position 94, Q by N at position 94, Q by R at  
15 position 94, Q by S at position 94, Q by T at position 94, I by D at  
position 95, I by E at position 95, I by K at position 95, I by N at position  
95, I by Q at position 95, I by R at position 95, I by S at position 95, I by  
T at position 95, H by D at position 97, H by E at position 97, H by K at  
position 97, H by N at position 97, H by Q at position 97, H by R at  
20 position 97, H by S at position 97, H by T at position 97, L by D at  
position 98, L by E at position 98, L by K at position 98, L by N at  
position 98, L by Q at position 98, L by R at position 98, L by S at  
position 98, L by T at position 98, V by D at position 101, V by E at  
position 101, V by K at position 101, V by N at position 101, V by Q at  
25 position 101, V by R at position 101, V by S at position 101, V by T at  
position 101, M by C at position 1, L by C at position 6, Q by C at  
position 10, S by C at position 13, Q by C at position 16, L by C at  
position 17, V by C at position 101, L by C at position 98, H by C at



-252-

position 97, Q by C at position 94, V by C at position 91, N by C at position 90,

wherein residue 1 corresponds to residue 1 of the mature IFN $\beta$  cytokine set forth in SEQ ID NOS:196.

- 5        373. A modified IFN $\beta$  cytokine selected from the group consisting of proteins comprising one or more single amino acid replacements in SEQ ID NOS:196, or any combination thereof, corresponding to the replacement of:
- D by Q at position 39, D by H at position 39, D by G at position 39, E by  
10 Q at position 42, E by H at position 42, K by Q at position 45, K by T at position 45, K by S at position 45, K by H at position 45, L by V at position 47, L by I at position 47, L by T at position 47, L by Q at position 47, L by H at position 47, L by A at position 47, K by Q at position 52, K by T at position 52, K by S at position 52, K by H at  
15 position 52, F by I at position 67, F by V at position 67, R by H at position 71, R by Q at position 71, D by H at position 73, D by G at position 73, D by Q at position 73, E by Q at position 81, E by H at position 81, E by Q at position 107, E by H at position 107, K by Q at position 108, K by T at position 108, K by S at position 108, K by H at  
20 position 108, E by Q at position 109, E by H at position 109, D by Q at position 110, D by H at position 110, D by G at position 110, F by I at position 111, F by V at position 111, R by H at position 113, R by Q at position 113, L by V at position 116, L by I at position 116, L by T at position 116, L by Q at position 116, L by H at position 116, L by A at  
25 position 116, L by V at position 120, L by I at position 120, L by T at position 120, L by Q at position 120, L by H at position 120, L by A at position 120, K by Q at position 123, K by T at position 123, K by S at position 123, K by H at position 123, R by H at position 124,, R by Q at position 124, R by H at position 128, R by Q at position 128, L by V at

-253-

position 130, L by I at position 130, L by T at position 130, L by Q at position 130, L by H at position 130, L by A at position 130, K by Q at position 134, K by T at position 134, K by S at position 134, K by H at position 134, K by Q at position 136, K by T at position 136, K by S at  
 5 position 136,, K by H at position 136, E by Q at position 137, E by H at position 137, Y by H at position 163, Y by I at position 163, R by H at position 165, R by Q at position 165, wherein the first amino acid listed is substituted by the second at the position indicated.

374. A modified IFN $\beta$  cytokine selected from the group consisting  
 10 of proteins comprising one or more single amino acid replacements in SEQ ID NOS:196, or any combination thereof, corresponding to the replacement of: M by V at position 1, M by I at position 1, M by T at position 1, M by Q at position 1, M by A at position 1, L by V at position 5, L by I at position 5, L by T at position 5, L by Q at position  
 15 5, L by H at position 5, L by A at position 5, F by I at position 8, F by V at position 8, L by V at position 9, L by I at position 9, L by T at position 9, L by Q at position 9, L by H at position 9, L by A at position 9, R by H at position 11, R by Q at position 11, F by I at position 15, F by V at position 15, K by Q at position 19, K by T at position 19, K by S  
 20 at position 19, K by H at position 19, W by S at position 22, W by H at position 22, N by H at position 25, N by S at position 25, N by Q at position 25, R by H at position 27, R by Q at position 27, L by V at position 28, L by I at position 28, L by T at position 28, L by Q at position 28, L by H at position 28, L by A at position 28, E by Q at position 29, E by H  
 25 at position 29, Y by H at position 30, Y by I at position 30, L by V at position 32, L by I at position 32, L by T at position 32, L by Q at position 32, L by H at position 32, L by A at position 32, K by Q at position 33, K by T at position 33, K by S at position 33, K by H at position 33, R by H at position 35, R by Q at position 35, M by V at

-254-

position 36, M by I at position 36, M by T at position 36, M by Q at  
position 36, M by A at position 36, D by Q at position 39, D by H at  
position 39, D by G at position 39, E by Q at position 42, E by H at  
position 42, K by Q at position 45, K by T at position 45, K by S at  
5 position 45, K by H at position 45, L by V at position 47, L by I at  
position 47, L by T at position 47, L by, Q at position 47, L by H at  
position 47, L by A at position 47, K by Q at position 52, K by T at  
position 52, K by S at position 52, K by H at position 52, F by I at  
position 67, F by V at position 67, R by H at position 71, R by Q at  
10 position 71, D by Q at position 73, D by H at position 73, D by G at  
position 73, E by Q at position 81, E by H at position 81, E by Q at  
position 85, E by H at position 85, Y by H at position 92, Y by I at  
position 92, K by Q at position 99, K by T at position 99, K by S at  
position 99, K by H at position 99, E by Q at position 103, E by H at  
15 position 103, E by Q at position 104, E by H at position 104, K by Q at  
position 105, K by T at position 105, K by S at position 105, K by H at  
position 105, E by Q at position 107, E by H at position 107, K by Q at  
position 108, K by T at position 108, K by S at position 108, K by H at  
position 108, E by Q at position 109, E by H at position 109, D by Q at  
20 position 110, D by H at position 110, D by G at position 110, F by I at  
position 111, F by V at position 111, R by H at position 113, R by Q at  
position 113, L by V at position 116, L by I at position 116, L by T at  
position 116, L by Q at position 116, L by H at position 116, L by A at  
position 116, L by V at position 120, L by I at position 120, L by T at  
25 position 120, L by Q at position 120, L by H at position 120, L by A at  
position 120, K by Q at position 123, K by T at position 123, K by S at  
position 123, K by H at position 123, R by H at position 124, R by Q at  
position 124, R by H at position 128, R by Q at position 128, L by V at  
position 130, L by I at position 130, L by T at position 130, L by Q at

-255-

position 130, L by H at position 130, L by A at position 130, K by Q at  
position 134, K by T at position 134, K by S at position 134, K by H at  
position 134, K by Q at position 136, K by T at position 136, K by S at  
position 136, K by H at position 136, E by Q at position 137, E by H at  
5 position 137, Y by H at position 138, Y by I at position 138, R by H at  
position 152, R by Q at position 152, Y by H at position 155, Y by I at  
position 155, R by H at position 159, R by Q at position 159, Y by H at  
position 163, Y by I at position 163, R by H at position 165, R by Q at  
position 165, M by D at position 1, M by E at position 1, M by K at  
10 position 1, M by N at position 1, M by R at position 1, M by S at position  
1, L by D at position 5, L by E at position 5, L by K at position 5, L by N  
at position 5, L by R at position 5, L by S at position 5, L by D at position  
6, L by E at position 6, L by K at position 6, L by N at position 6, L by R  
at position 6, L by S at position 6, L by Q at position 6, L by T at position  
15 6, F by E at position 8, F by K at position 8, F by R at position 8, F by D  
at position 8, L by D at position 9, L by E at position 9, L by K at position  
9, L by N at position 9, L by R at position 9, L by S at position 9, Q by D  
at position 10, Q by E at position 10, Q by K at position 10, Q by N at  
position 10, Q by R at position 10, Q by S at position 10, Q by T at  
20 position 10, S by D at position 12, S by E at position 12, S by K at  
position 12, S by R at position 12, S by D at position 13, S by E at  
position 13, S by K at position 13, S by R at position 13, S by N at  
position 13, S by Q at position 13, S by T at position 13, N by D at  
position 14, N by E at position 14, N by K at position 14, N by Q at  
25 position 14, N by R at position 14, N by S at position 14, N by T at  
position 14, F by D at position 15, F by E at position 15, F by K at  
position 15, F by R at position 15, Q by D at position 16, Q by E at  
position 16, Q by K at position 16, Q by N at position 16, Q by R at  
position 16, Q by S at position 16, Q by T at position 16, C by D at

-256-

position 17, C by E at position 17, C by K at position 17, C by N at  
position 17, C by Q at position 17, C by R at position 17, C by S at  
position 17, C by T at position 17, L by N at position 20, L by Q at  
position 20, L by R at position 20, L by S at position 20, L by T at  
5 position 20, L by D at position 20, L by E at position 20, L by K at  
position 20, W by D at position 22, W by E at position 22, W by K at  
position 22, W by R at position 22, Q by D at position 23, Q by E at  
position 23, Q by K at position 23, Q by R at position 23, L by D at  
position 24, L by E at position 24, L by K at position 24, L by R at  
10 position 24, W by D at position 79, W by E at position 79, W by K at  
position 79, W by R at position 79, N by D at position 80, N by E at  
position 80, N by K at position 80, N by R at position 80, T by D at  
position 82, T by E at position 82, T by K at position 82, T by R at  
position 82, I by D at position 83, I by E at position 83, I by K at position  
15 83, I by R at position 83, I by N at position 83, I by Q at position 83, I by  
S at position 83, I by T at position 83, N by D at position 86, N by E at  
position 86, N by K at position 86, N by R at position 86, N by Q at  
position 86, N by S at position 86, N by T at position 86, L by D at  
position 87, L by E at position 87, L by K at position 87, L by R at  
20 position 87, L by N at position 87, L by Q at position 87, L by S at  
position 87, L by T at position 87, A by D at position 89, A by E at  
position 89, A by K at position 89, A by R at position 89, N by D at  
position 90, N by E at position 90, N by K at position 90, N by Q at  
position 90, N by R at position 90, N by S at position 90, N by T at  
25 position 90, V by D at position 91, V by E at position 91, V by K at  
position 91, V by N at position 91, V by Q at position 91, V by R at  
position 91, V by S at position 91, V by T at position 91, Q by D at  
position 94, Q by E at position 94, Q by Q at position 94, Q by N at  
position 94, Q by R at position 94, Q by S at position 94, Q by T at

-257-

position 94, I by D at position 95, I by E at position 95, I by K at position  
95, I by N at position 95, I by Q at position 95, I by R at position 95, I by  
S at position 95, I by T at position 95, H by D at position 97, H by E at  
position 97, H by K at position 97, H by N at position 97, H by Q at  
5 position 97, H by R at position 97, H by S at position 97, H by T at  
position 97, L by D at position 98, L by E at position 98, L by K at  
position 98, L by N at position 98, L by Q at position 98, L by R at  
position 98, L by S at position 98, L by T at position 98, V by D at  
position 101, V by E at position 101, V by K at position 101, V by N at  
10 position 101, V by Q at position 101, V by R at position 101, V by S at  
position 101, V by T at position 101, M by C at position 1, L by C at  
position 6, Q by C at position 10, S by C at position 13, Q by C at  
position 16, L by C at position 17, V by C at position 101, L by C at  
position 98, H by C at position 97, Q by C at position 94, V by C at  
15 position 91, N by C at position 90, D by Q at position 39, D by H at  
position 39, D by G at position 39, E by Q at position 42, E by H at  
position 42, K by Q at position 45, K by T at position 45, K by S at  
position 45, K by H at position 45, L by V at position 47, L by I at  
position 47, L by T at position 47, L by Q at position 47, L by H at  
20 position 47, L by A at position 47, K by Q at position 52, K by T at  
position 52, K by S at position 52, K by H at position 52, F by I at  
position 67, F by V at position 67, R by H at position 71, R by Q at  
position 71, D by H at position 73, D by G at position 73, D by Q at  
position 73, E by Q at position 81, E by H at position 81, E by Q at  
25 position 107, E by H at position 107, K by Q at position 108, K by T at  
position 108, K by S at position 108, K by H at position 108, E by Q at  
position 109, E by H at position 109, D by Q at position 110, D by H at  
position 110, D by G at position 110, F by I at position 111, F by V at  
position 111, R by H at position 113, R by Q at position 113, L by V at

-258-

position 116, L by I at position 116, L by T at position 116, L by Q at  
position 116, L by H at position 116, L by A at position 116, L by V at  
position 120, L by I at position 120, L by T at position 120, L by Q at  
position 120, L by H at position 120, L by A at position 120, K by Q at  
5 position 123, K by T at position 123, K by S at position 123, K by H at  
position 123, R by H at position 124,, R by Q at position 124, R by H at  
position 128, R by Q at position 128, L by V at position 130, L by I at  
position 130, L by T at position 130, L by Q at position 130, L by H at  
position 130, L by A at position 130, K by Q at position 134, K by T at  
10 position 134, K by S at position 134, K by H at position 134, K by Q at  
position 136, K by T at position 136, K by S at position 136,, K by H at  
position 136, E by Q at position 137, E by H at position 137, Y by H at  
position 163, Y by I at position 163I, R by H at position 165, R by Q at  
position 165, wherein the first amino acid listed is substituted by the  
15 second at the position indicated.

375. A modified IFN $\beta$  of claim 374 selected from the group  
consisting of a modified IFN $\beta$  set forth in any of SEQ ID Nos.234-289,  
989-1302.

IFN $\alpha$ -2b  
ETIPVLHEMQQIFNLFSTKDDSSAAWDETLLDKFYTETYQQQLNDLEACVI  
51 60 70 80 90 100

151 . 160

IFN $\alpha$ -2b FSLSTNLQESLSRSKE

FIG. 1A



Three dimensional structure of  $\text{INF}\alpha\text{-2b}$   
showing candidate LEADs

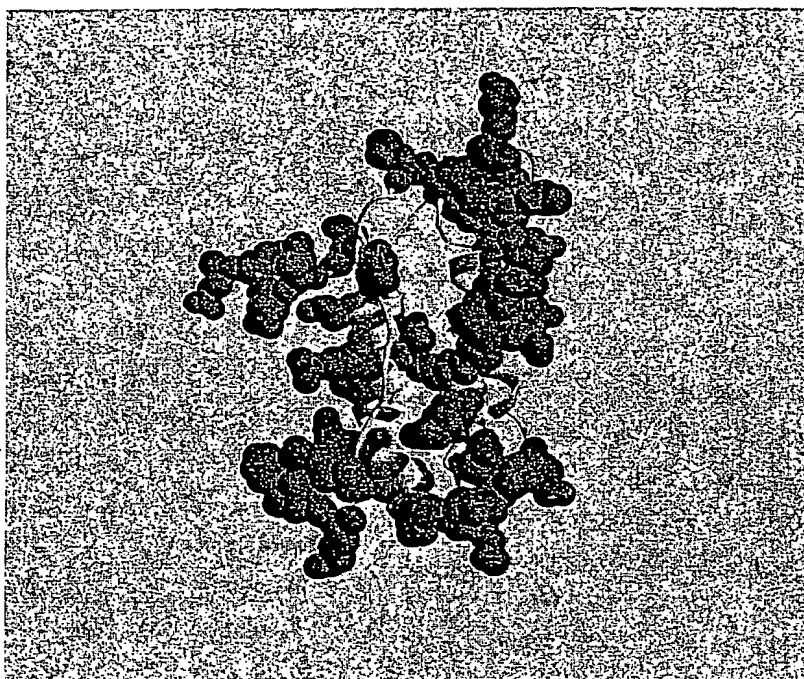


FIG.1B

The "Percent Accepted Mutation" (PAM250) matrix

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	V	Y	W
A	0	-2	0	0	-2	0	0	1	-1	-1	-2	-1	-1	-3	1	1	1	-6	-3	0
R	-2	6	0	-1	-4	1	-1	-3	2	-2	-3	3	0	-4	0	0	-1	2	-4	-2
N	0	0	2	2	-4	1	1	0	2	-2	-3	1	-2	-3	0	1	0	-4	-2	-2
D	0	-1	2	4	-5	2	3	1	1	-2	-4	0	-3	-6	-1	0	0	-7	-4	-2
C	-2	-4	-4	-5	12	-5	-5	-3	-3	-2	-6	-5	-5	-4	-3	0	-2	-8	0	-2
Q	0	1	1	2	-5	1	2	-1	3	-2	-2	1	-1	-5	0	-1	-1	-5	-4	-2
E	0	-1	1	3	-5	2	4	0	1	-2	-3	0	-2	-5	-1	0	0	-7	-4	-2
G	1	-3	0	1	-3	-1	0	5	-2	-3	-4	-2	-3	-5	0	1	0	-7	-5	-1
H	-1	2	2	1	-3	3	1	-2	6	-2	-2	0	-2	-2	0	-1	-1	-3	0	-2
I	-1	-2	-2	-2	-2	-2	-2	-3	-2	5	2	-2	2	1	-2	-1	0	-5	-1	4
L	-2	-3	-3	-4	-6	-2	-3	-4	-2	2	6	-3	4	2	-3	-3	-2	-2	-1	2
K	-1	3	1	0	-5	1	0	-2	0	-2	-3	5	0	-5	-1	0	0	-3	-4	-2
M	-1	0	-2	-3	-5	-1	-2	-3	-2	2	4	0	6	0	-2	-2	-1	-4	-2	2
F	-3	-4	-3	-6	-4	-5	-5	-5	-2	1	2	-5	0	9	-5	-3	-3	0	7	-1
P	1	0	0	-1	-3	0	-1	0	0	-2	-3	-1	-2	-5	6	1	0	-6	-5	-1
S	1	0	1	0	0	-1	0	1	-1	-1	-3	0	-2	-3	1	2	1	-2	-3	-1
T	1	-1	0	0	-2	-1	0	0	-1	0	-2	0	-1	-3	0	1	3	-5	-3	0
V	-6	2	-4	-7	-8	-5	-7	-7	-3	-5	-2	-3	-4	0	-6	-2	-5	17	0	-6
Y	-3	-4	-2	-4	0	-4	-4	-5	0	-1	-1	-4	-2	7	-5	-3	-3	0	10	-2
W	0	-2	-2	-2	-2	-2	-2	-1	-2	4	2	-2	2	-1	-1	-1	0	-6	-2	4

FIG.2

Scores from PAM250, given to residue substitutions to  
protect human INF  $\alpha$ -2b against proteolysis

	R	D	E	F	K	M	F	R	V	Y
A	-2	0	0	-2	-1	-1	-3	<b>1</b>	-6	-3
N	0	<b>2</b>	<b>1</b>	-3	<b>1</b>	-2	-3	0	-4	-2
C	-4	-5	-5	-6	-5	-5	-4	-3	-8	0
Q	<b>1</b>	<b>2</b>	<b>2</b>	-2	<b>1</b>	-1	-5	0	-5	-4
G	-3	1	0	-4	-2	-3	-5	0	-7	-5
H	<b>2</b>	1	<b>1</b>	-2	0	-2	-2	0	<b>-3</b>	<b>0</b>
I	-2	-2	-2	<b>2</b>	-2	<b>2</b>	<b>1</b>	-2	-5	<b>-1</b>
S	0	0	0	-3	0	-2	-3	<b>1</b>	<b>-2</b>	-3
T	-1	0	0	-2	0	-1	-3	0	-5	-3
V	-2	-2	-2	<b>2</b>	-2	<b>2</b>	<b>-1</b>	-1	-6	-2

FIG.3

Protection against proteolysis for interferon  $\alpha$ -2b variants

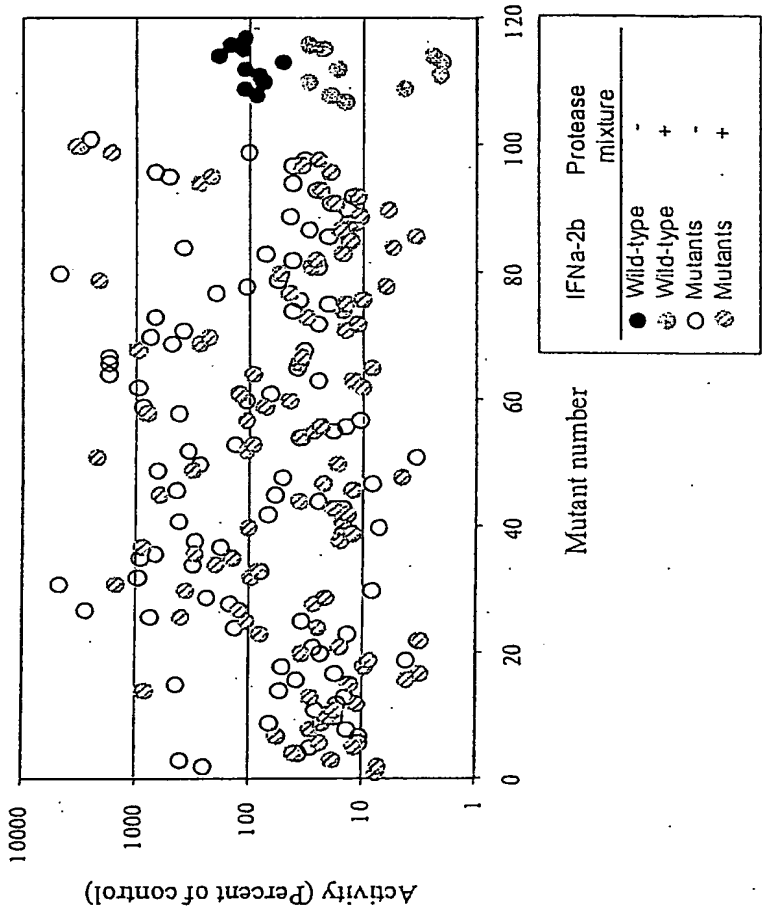


FIG.4A

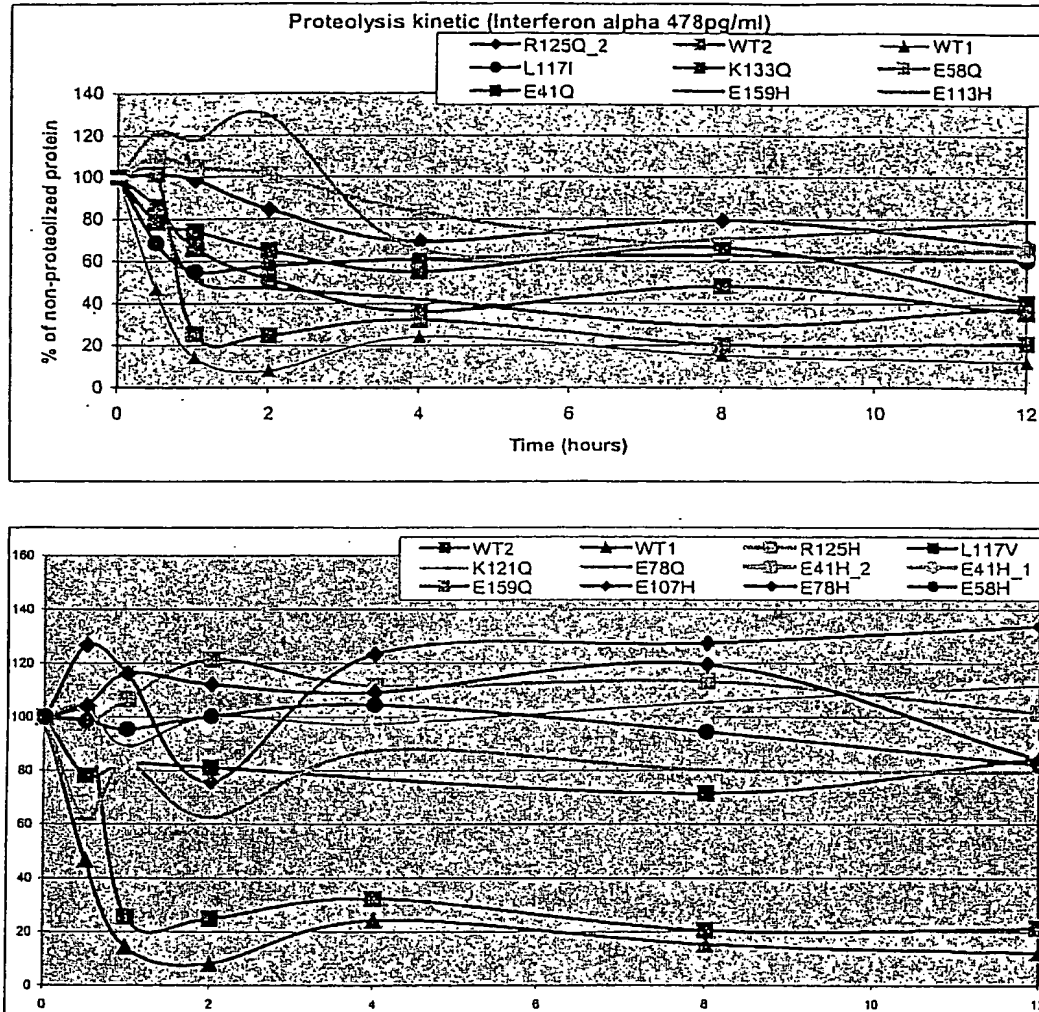


FIG.4B

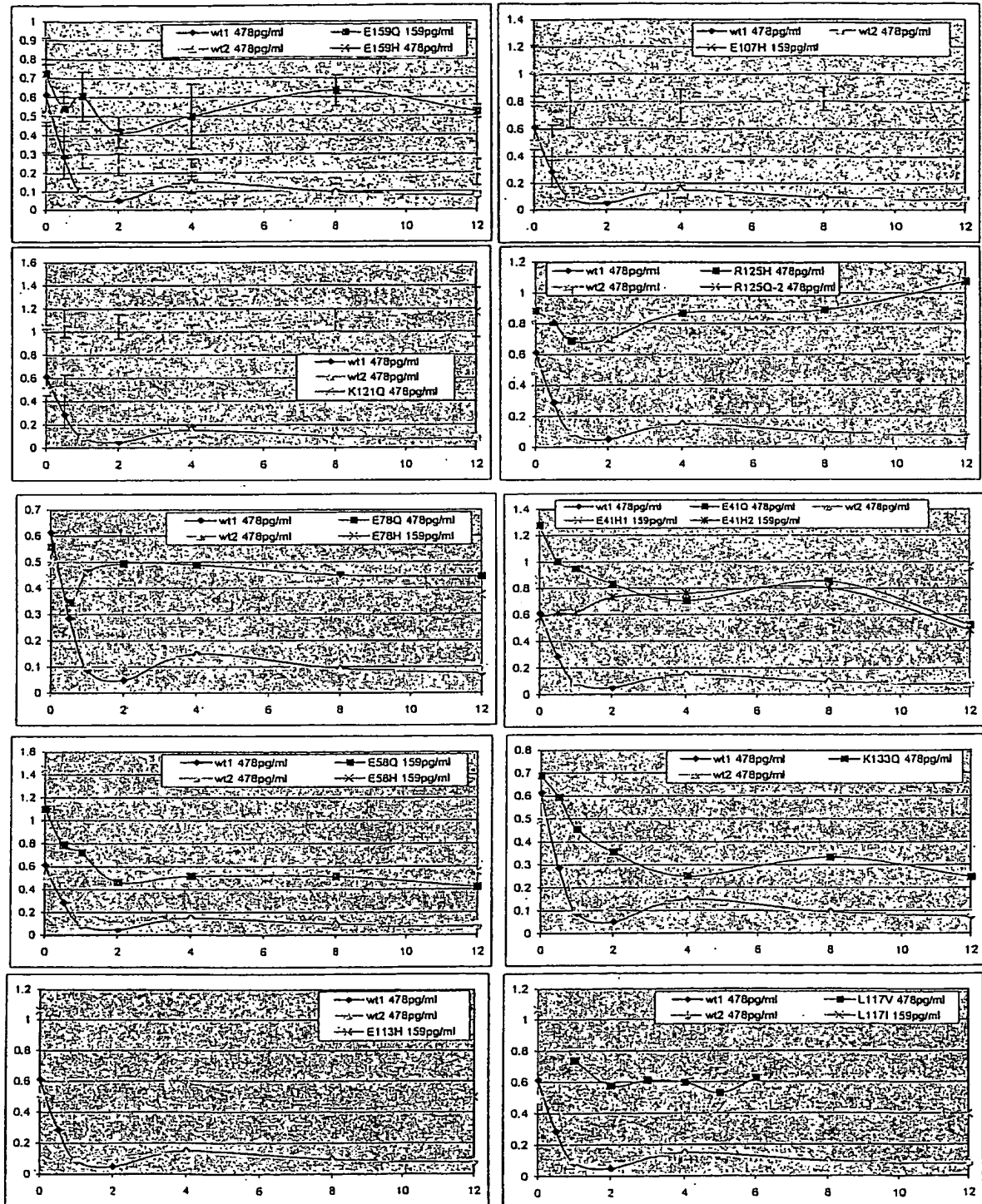


FIG.4C

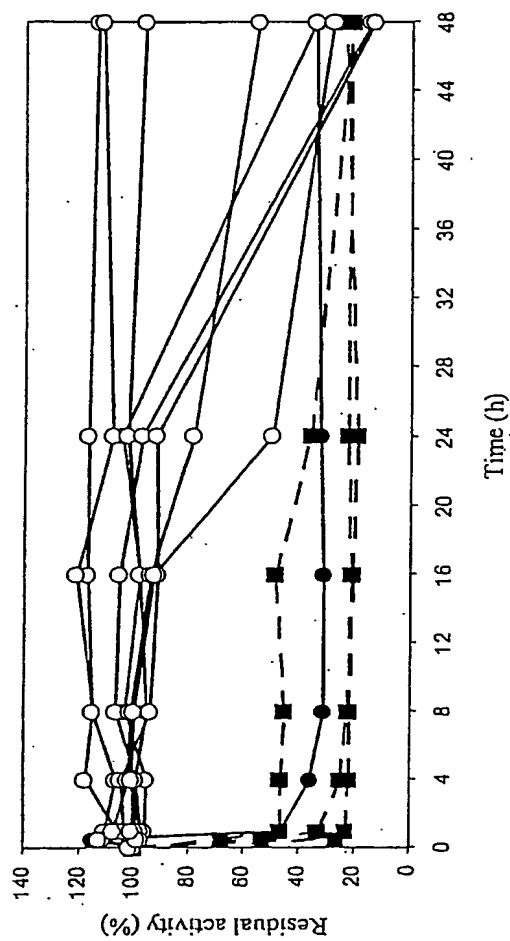
Treatment with chymotrypsin

FIG.5

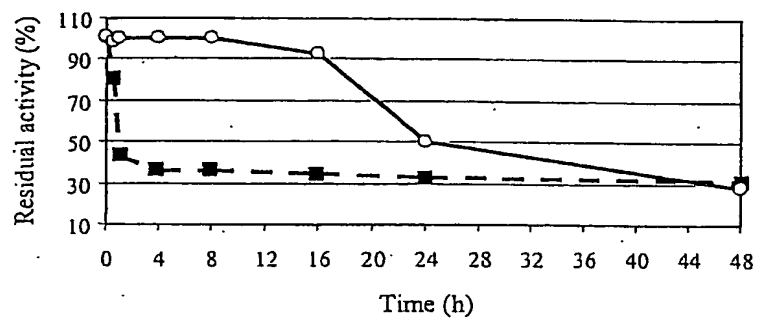
Treatment with chymotrypsin

FIG.6A

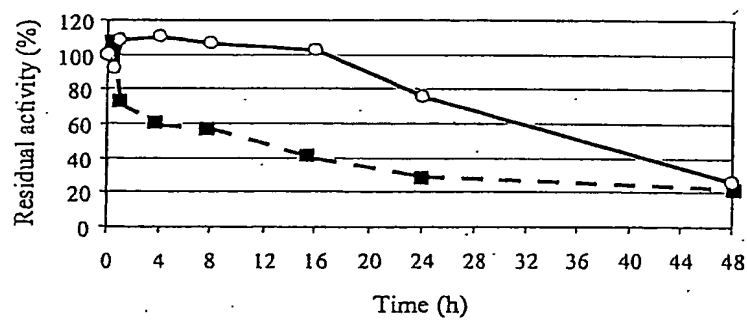
Treatment with protease mixture

FIG.6B



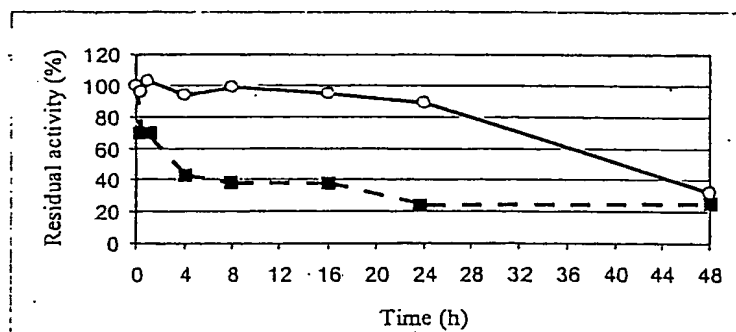
Treatment with blood lysate

FIG.6C

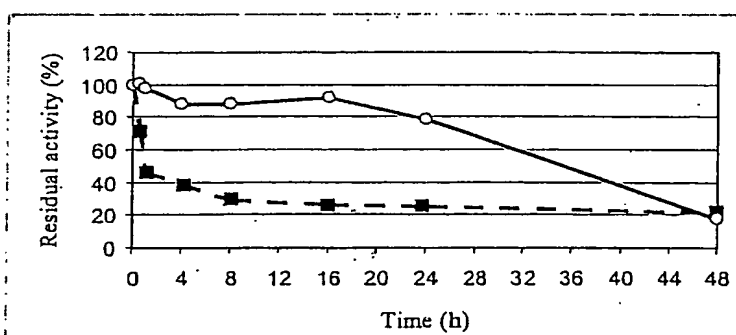
Treatment with serum

FIG.6D

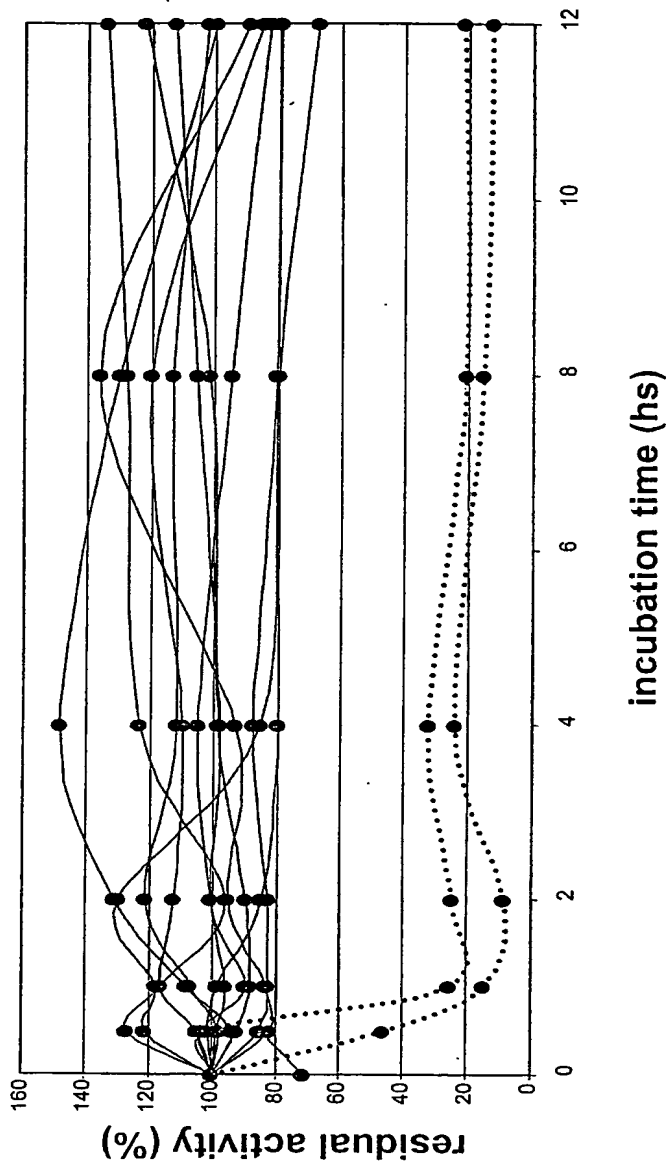


FIG. 6(E)

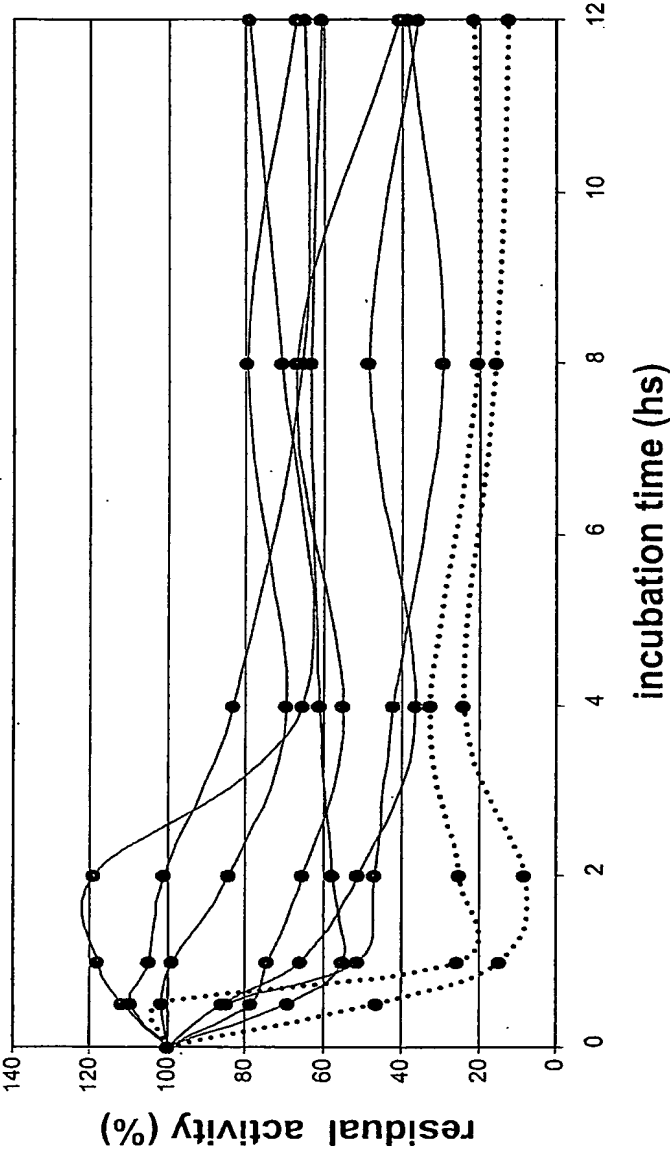


FIG. 6(F)

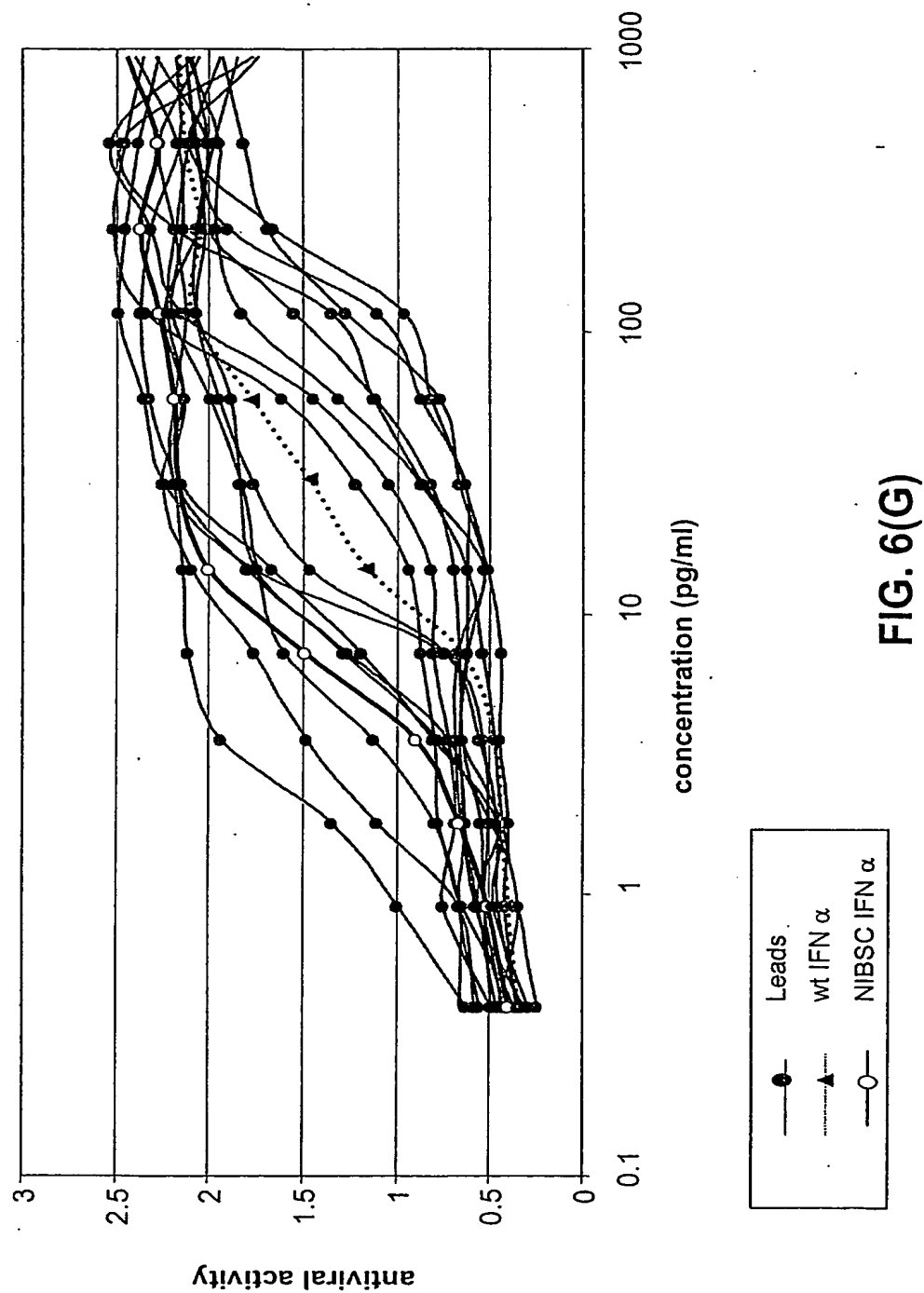


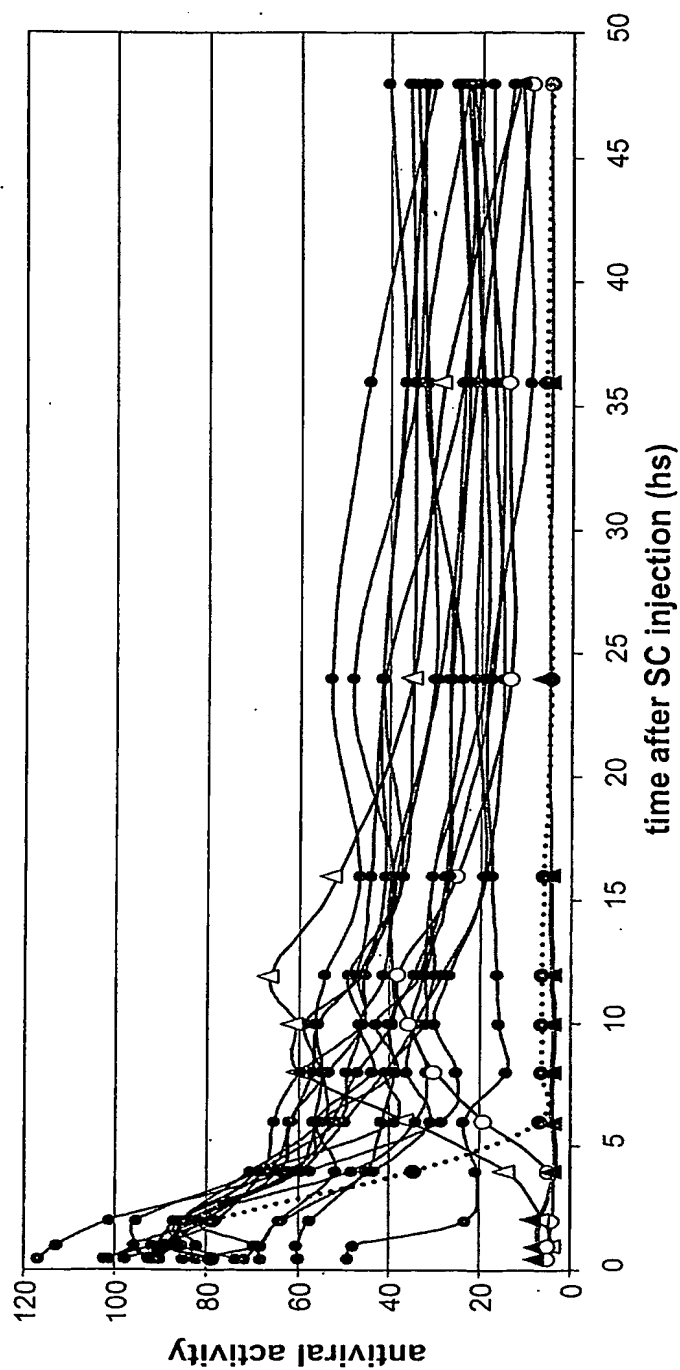
FIG. 6(G)

## Potency (antiproliferation) – IFN $\alpha$ leads

---

	Potency (10 <sup>8</sup> U/mg)
WT	1,70
Lead 13	1,60
Lead 9	1,90
Lead 8	2,05
Lead 2	3,70
Lead 16	1,60
Lead 4	0,50
Lead 5	0,65
Lead 15	3,20
Lead 10	0,50
Lead 12	1,50
Lead 11	nd
Lead 6	1,20
Lead 1	2,95
Lead 7	1,60
Lead 3	2,25
Lead 14	nd

Fig. 6(H)



Reference to Figures 6I, 6K-N

- Leads
- △ Pegasys 36  $\mu\text{g/ml}$
- wt IFN  $\alpha$
- Pegasys 18  $\mu\text{g/ml}$
- ▲ vehicle

FIG. 6(I)

## FIGURE 6(J)

### IFN- $\alpha$ LEADS

IFN- $\alpha$ LEAD	SEQ ID N°	Mutation(s)
1	983	K121Q / P109A
2	987	E159H / Y89H
3	124	E159Q
4	90	E58H
5	89	E58Q
6	979	E41H / Y89H / N45D
7	103	L117I
8	986	R125H / M111V
9	96	E107H
10	101	E113H
11	87	E41Q
12	107	R125Q
13	985	L117V / A139G
14	980	E41Q / D94G
15	93	E78H
16	984	K133Q / K121Q / P109A / G102R

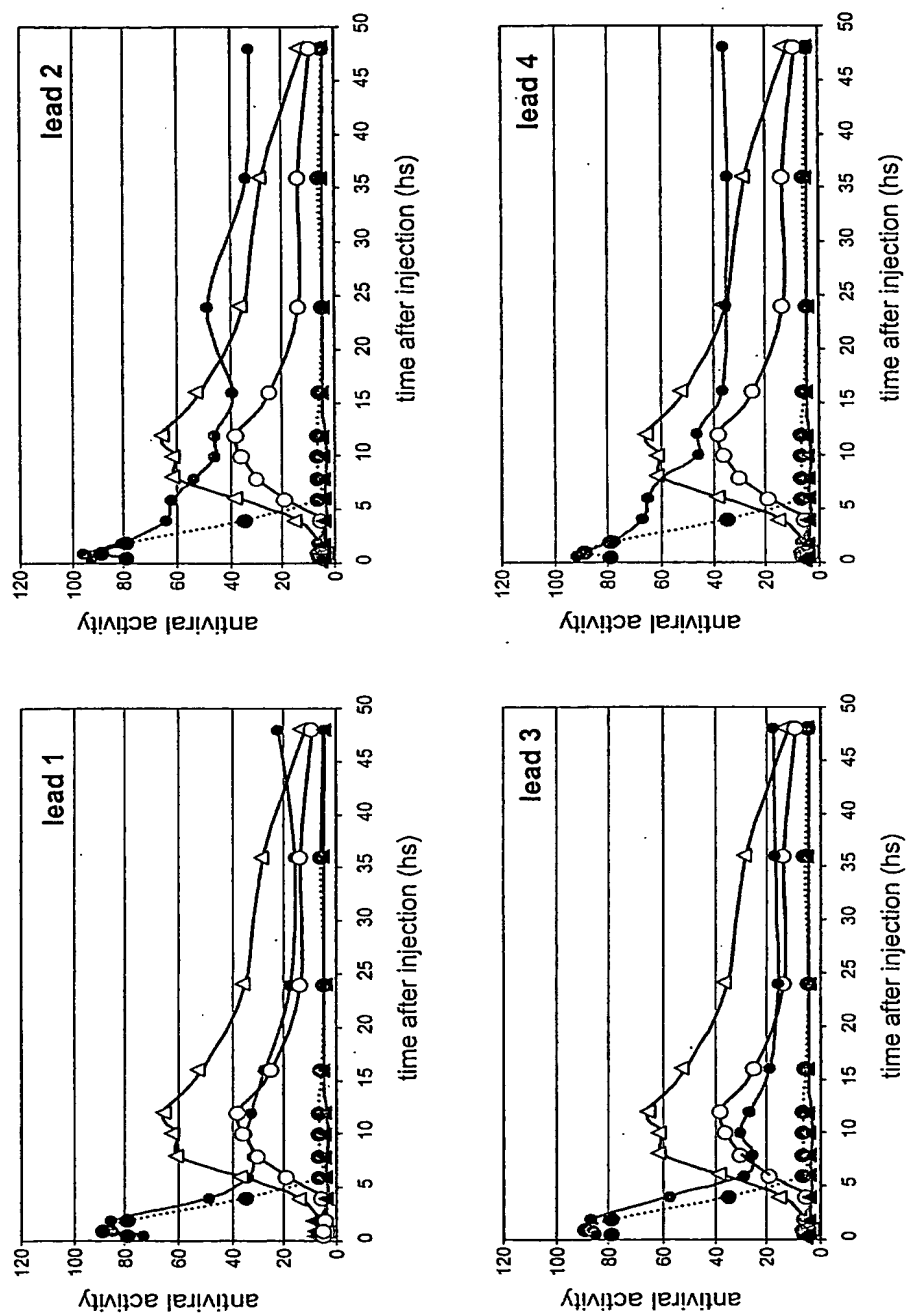


FIG. 6(K)



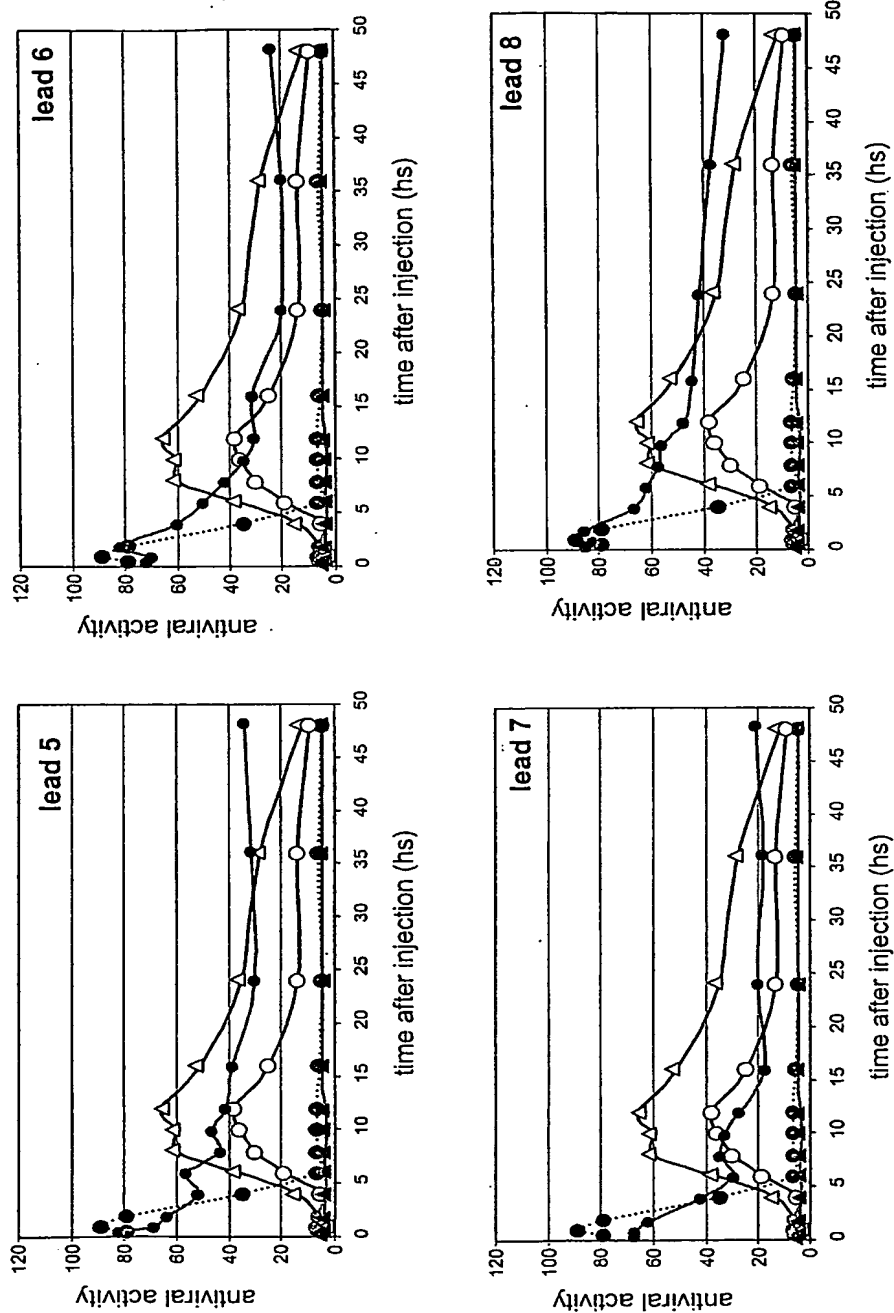


FIG. 6(L)

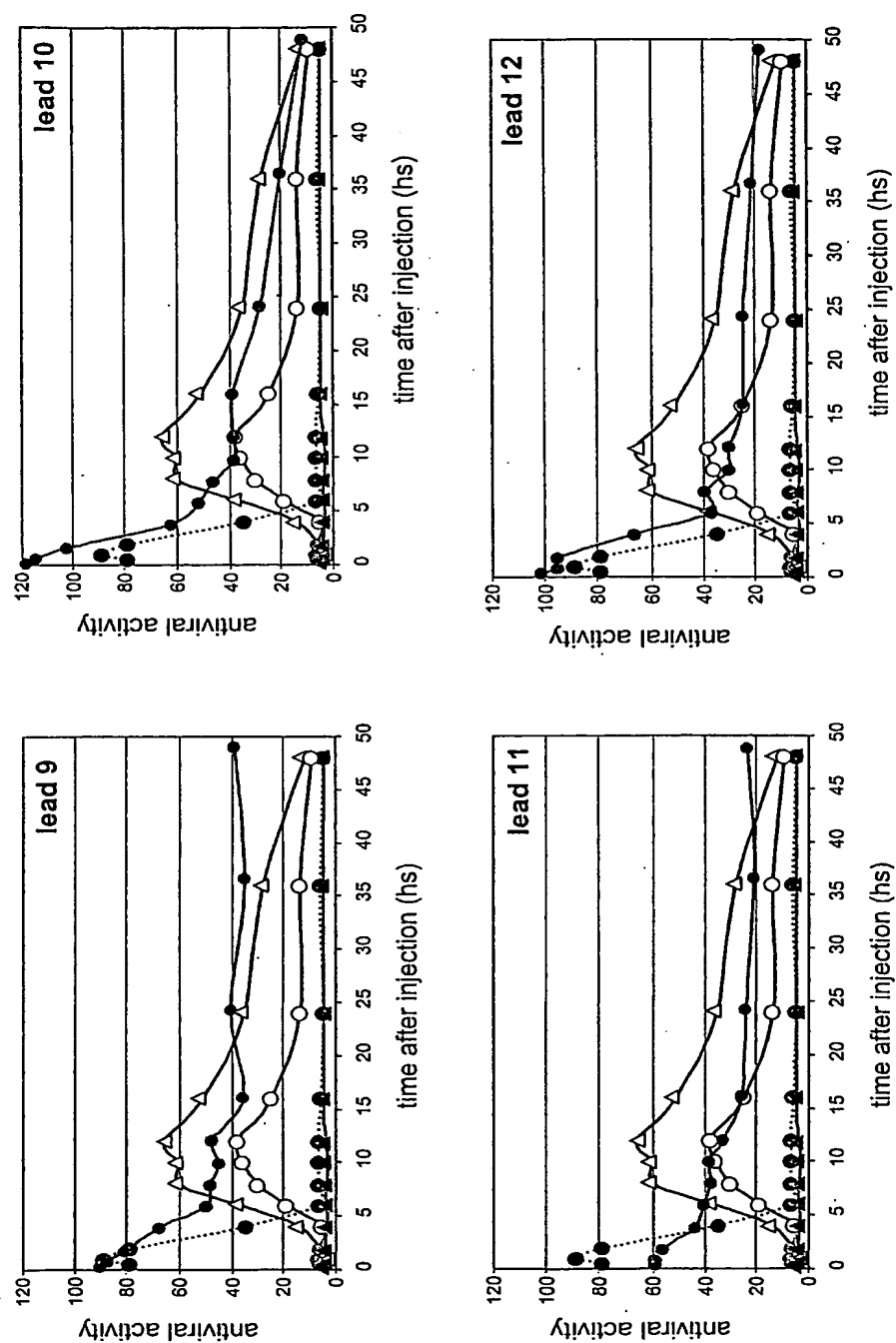


FIG. 6(M)

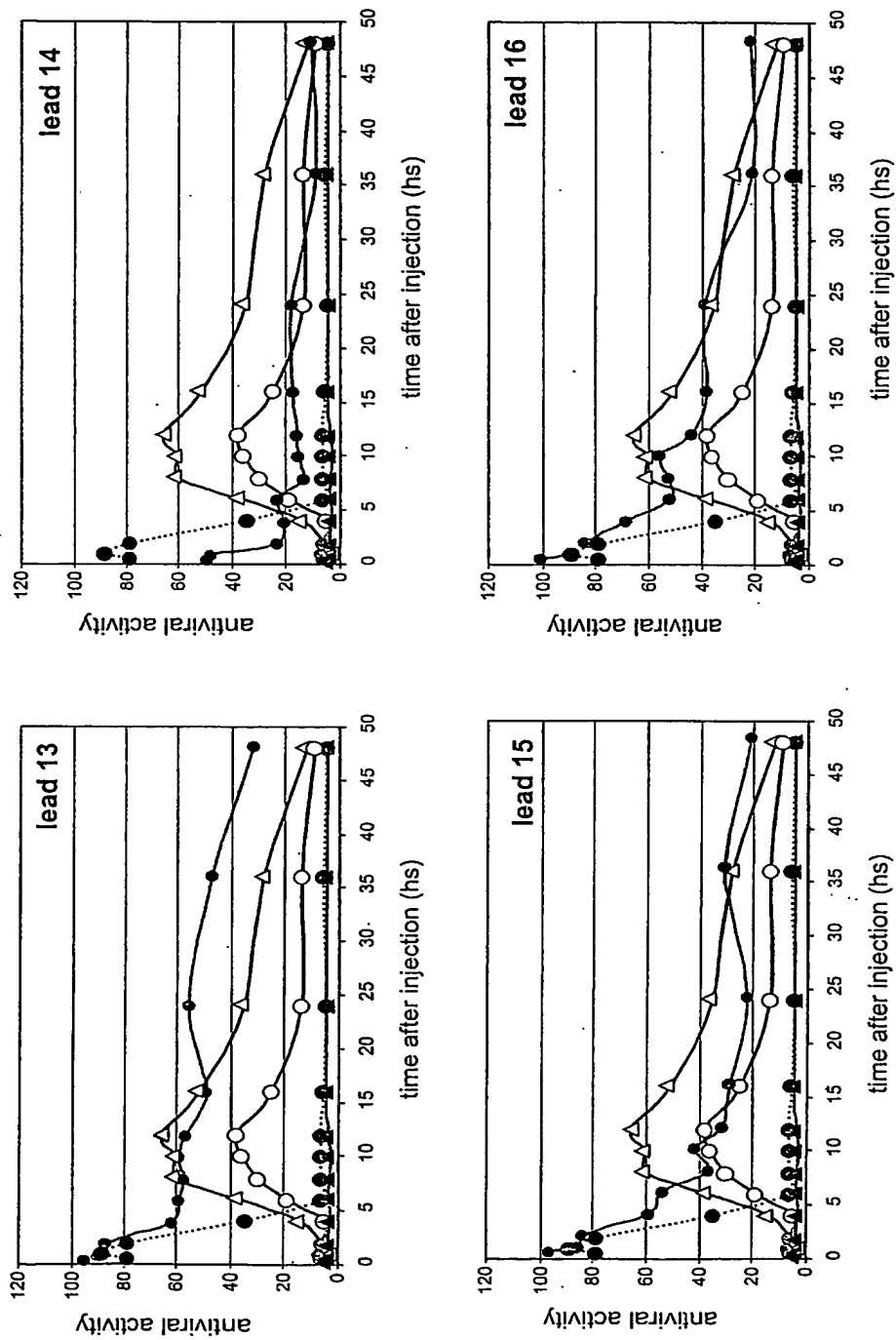
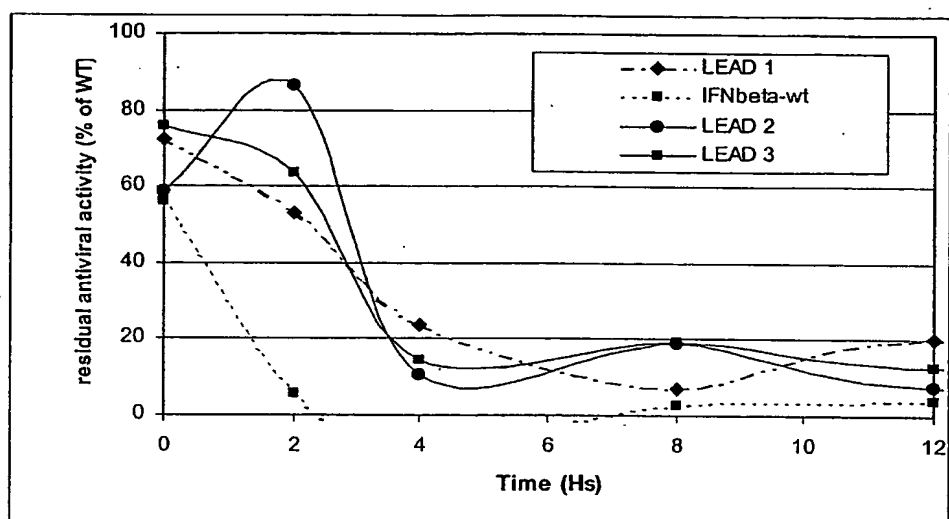


FIG. 6(N)

**FIG 6(O)**

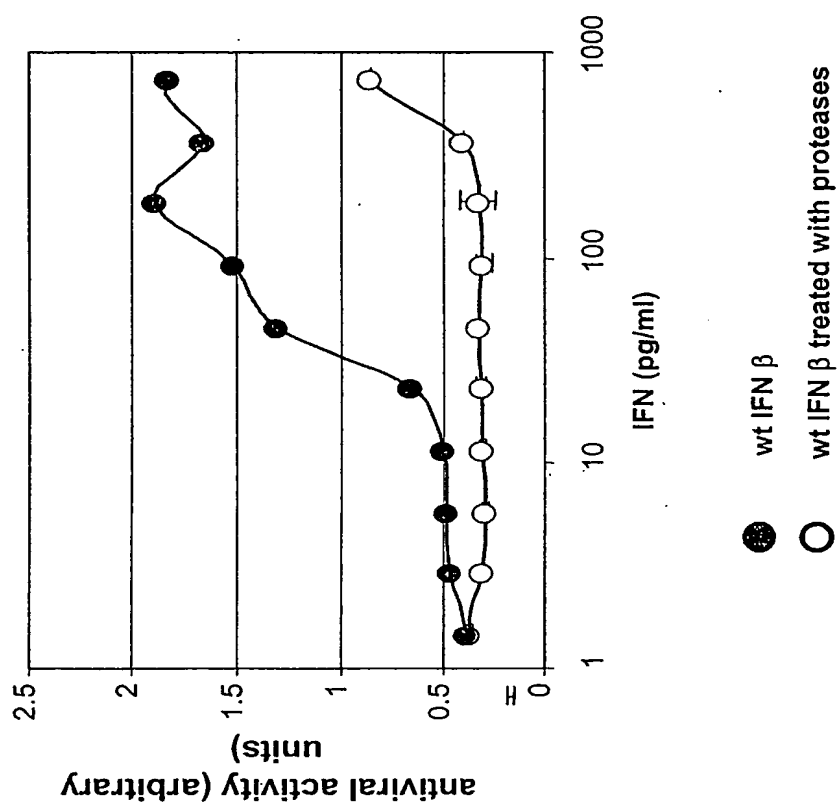


FIG. 6(P)

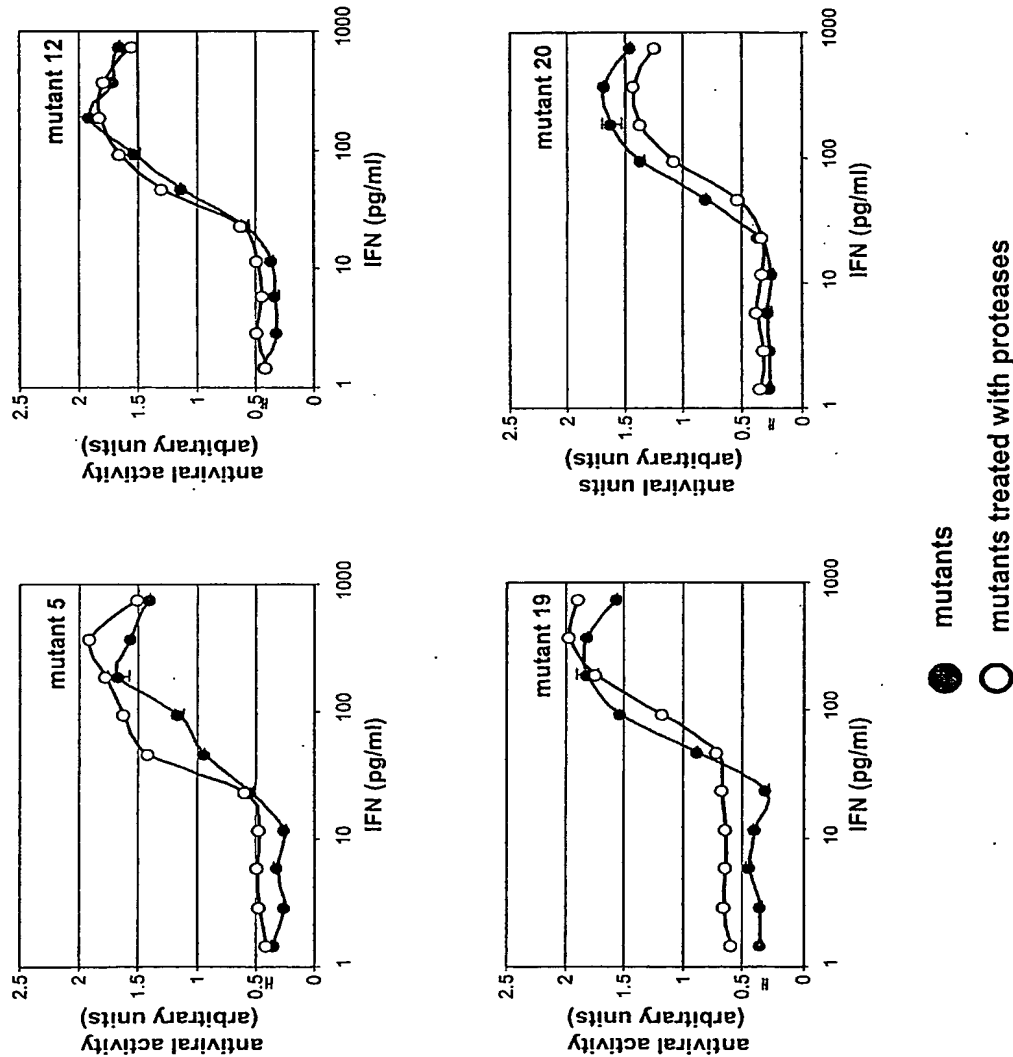


FIG. 6(Q)

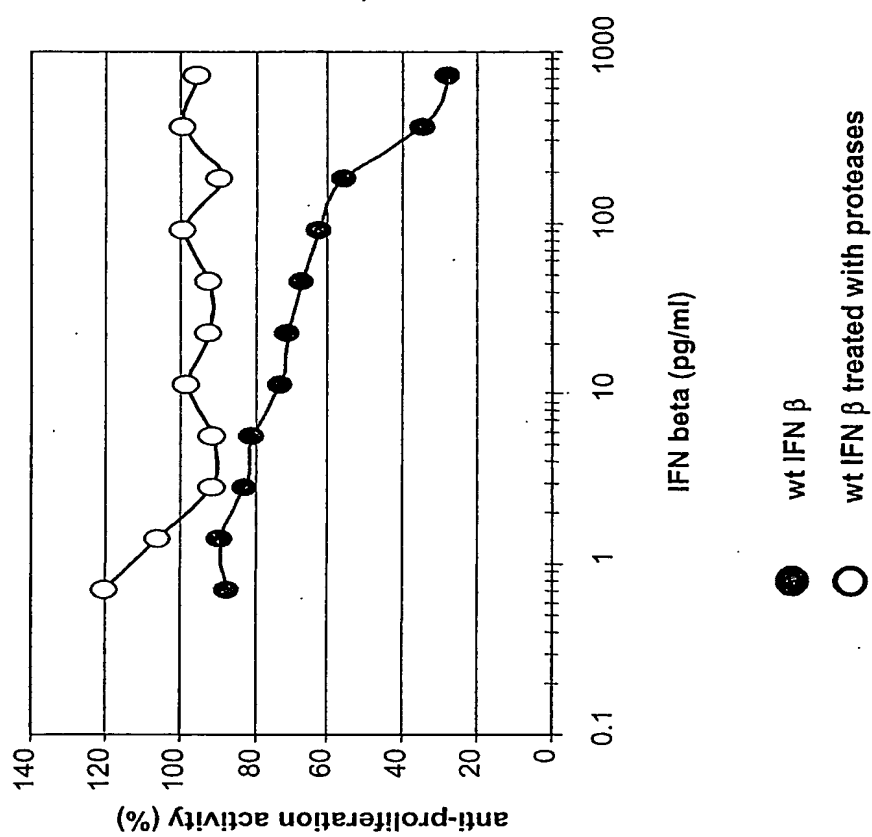


FIG. 6(R)

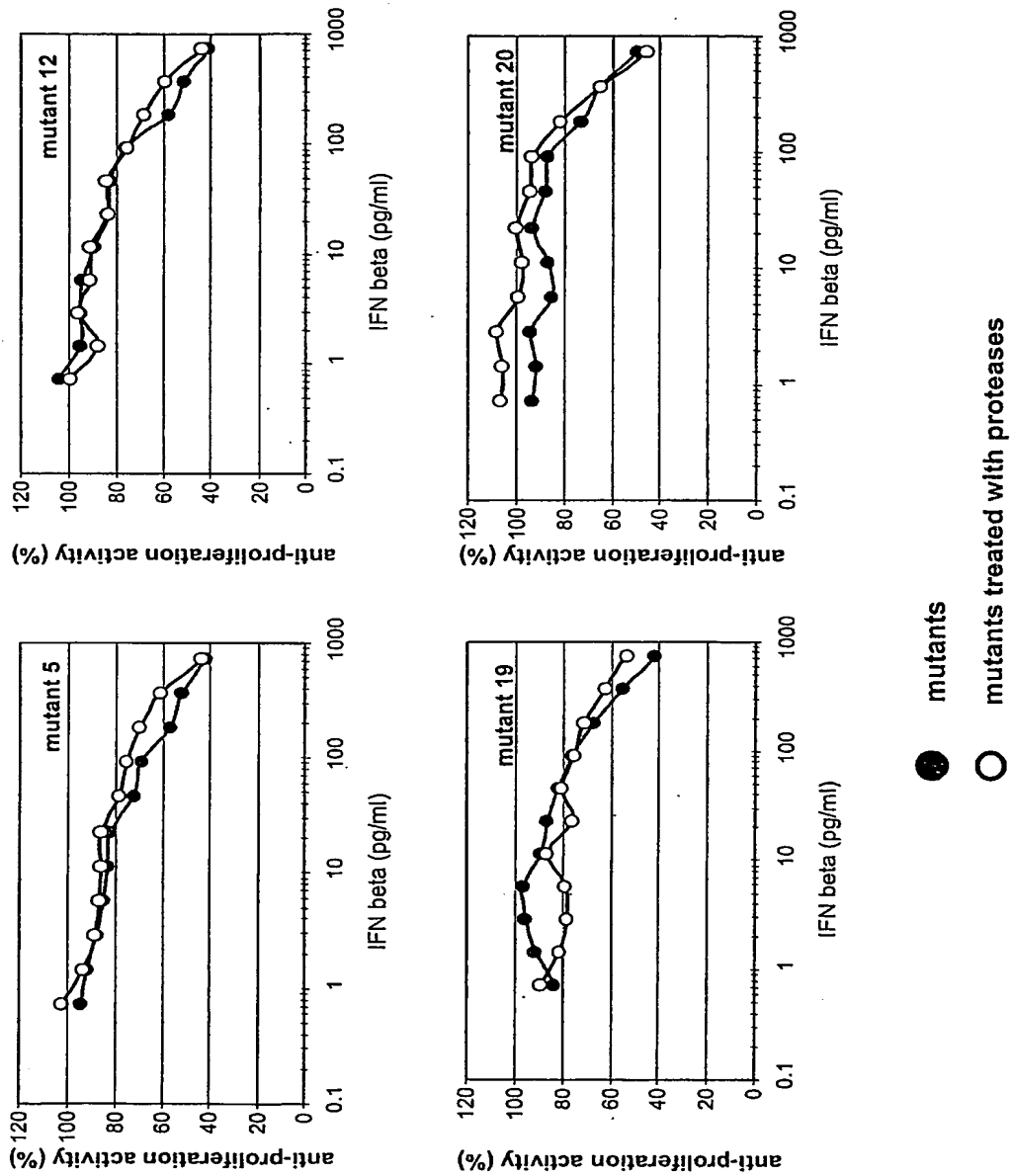


FIG. 6(S)



**Fig. 6(T) Summary – IFN $\alpha$  leads**

	Potency (AV) (10 <sup>8</sup> U/mg)	Potency (AP) (10 <sup>8</sup> U/mg)	AUC (arbitrary units)
WT	1,6	1,7	16,5
Pegasys			33,0
Pegasys			77,0
Lead 13	0,4	1,6	129,7
Lead 9	1,2	1,9	109,0
Lead 8	1,0	2,1	107,0
Lead 2	2,1	3,7	105,0
Lead 16	0,8	1,6	101,6
Lead 4	4,3	0,5	100,0
Lead 5	1,2	0,7	88,6
Lead 15	1,7	3,2	88,0
Lead 10	5,5	0,5	85,6
Lead 12	1,4	1,5	77,0
Lead 11	28,5	nd	69,0
Lead 6	1,7	1,2	64,2
Lead 1	1,9	3,0	58,5
Lead 7	1,7	1,6	56,5
Lead 3	1,7	2,3	54,6
Lead 14	0,9	nd	25,0

**Fig. 6(U) IFN $\alpha$  LEADS-- Area under the curve (AUC)**

	AUC (arbitrary units)	protein injected ( $\mu\text{g/ml}^*$ )	IFN units injected / ml ( $\times 10^6$ )
WT	16,5	2,5	2,0
Pegasys	33,0	18,0	
Pegasys	77,0	36,0	
Lead 13	129,7	10,3	2,0
Lead 9	109,0	3,5	2,0
Lead 8	107,0	4,2	2,0
Lead 2	105,0	2,0	2,0
Lead 16	101,6	5,4	2,0
Lead 4	100,0	1,0	2,0
Lead 5	88,6	3,6	2,0
Lead 15	88,0	2,4	2,0
Lead 10	85,6	1,0	2,0
Lead 12	77,0	3,0	2,0
Lead 11	69,0	0,2	2,0
Lead 6	64,2	3,4	2,0
Lead 1	58,5	2,1	2,0
Lead 7	56,5	2,4	2,0
Lead 3	54,6	2,5	2,0
Lead 14	25,0	2,0	2,0

Interferon  $\alpha$ -2b structure in "space filling" representation

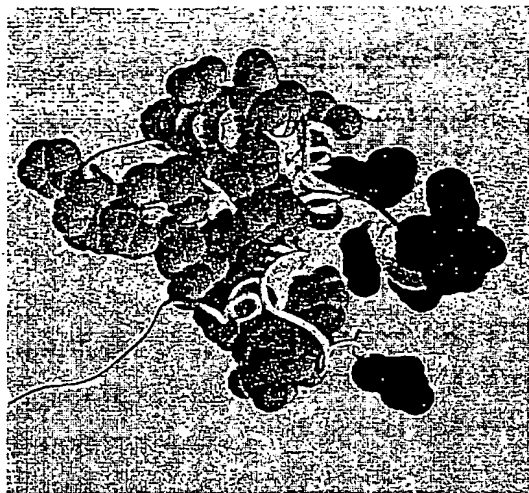


FIG.7A

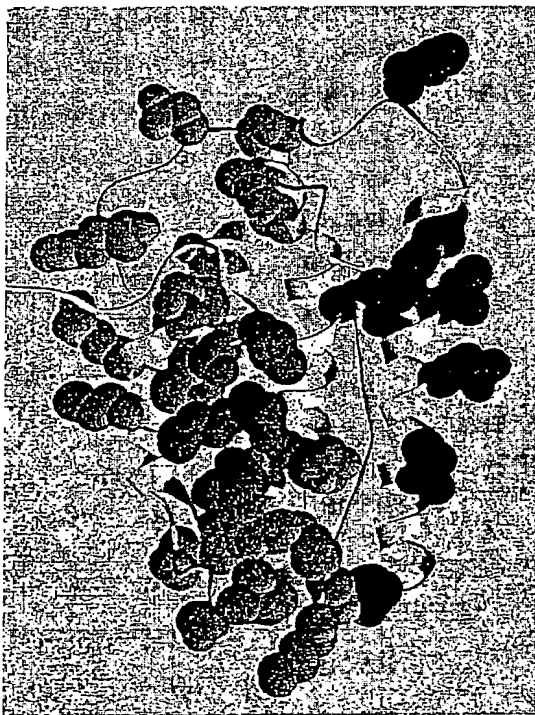


FIG.7B

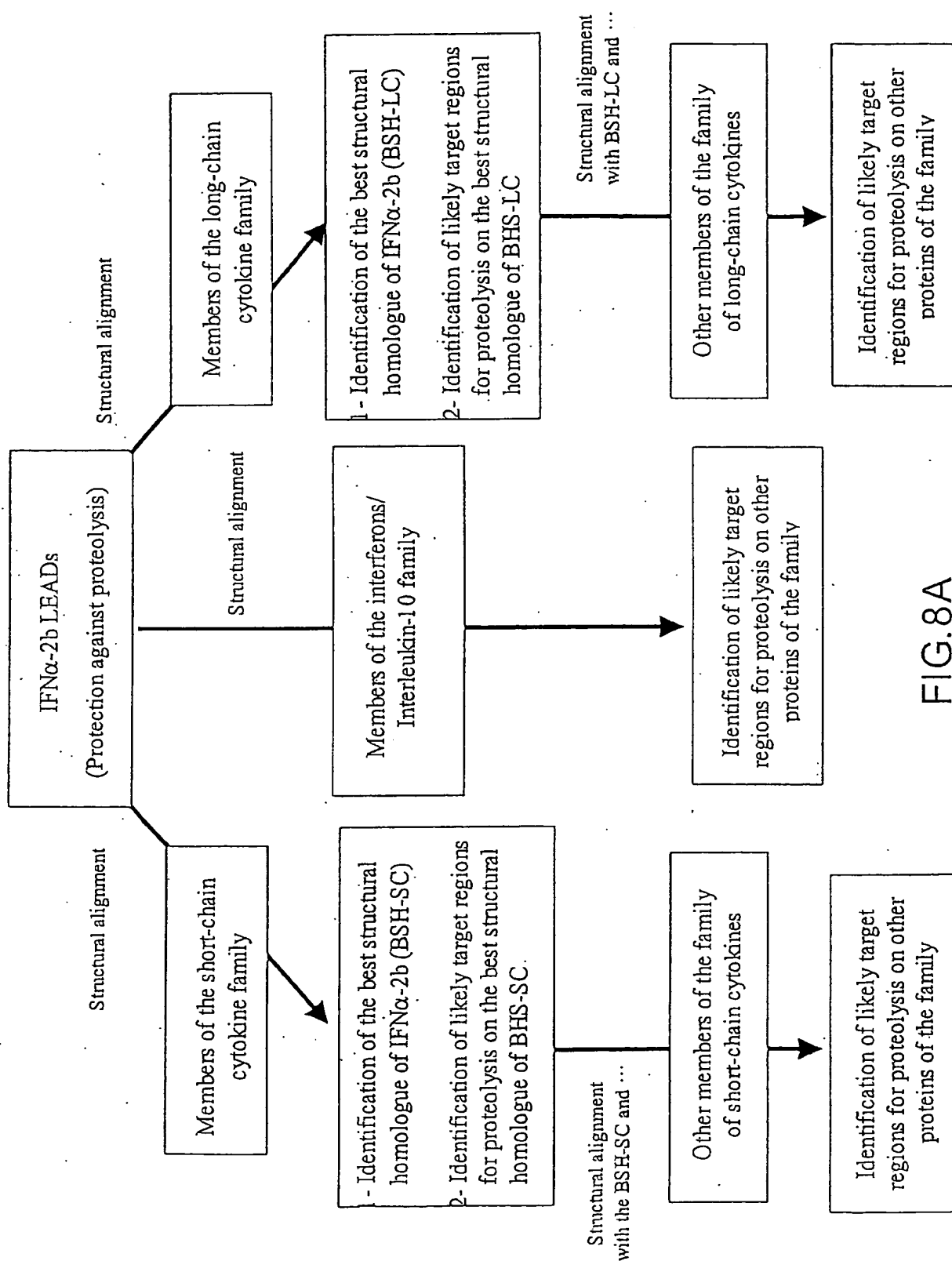


FIG.8A

Structural super-imposition of interferon  $\alpha$ -2b (1RH2)  
and interferon  $\beta$  (1AU1)

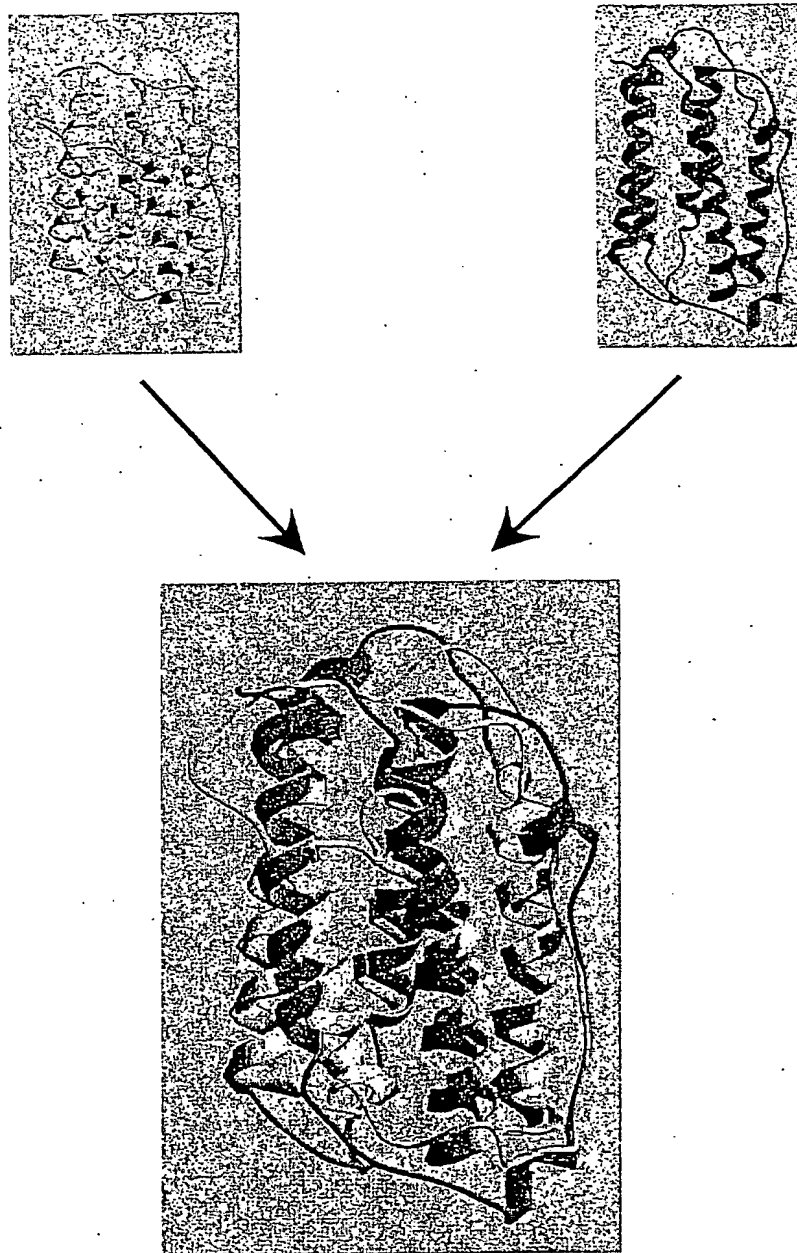


FIG.8B

Structural super-imposition of interferon  $\alpha$ -2b (1RH2)  
and erythropoietin (1BUY)

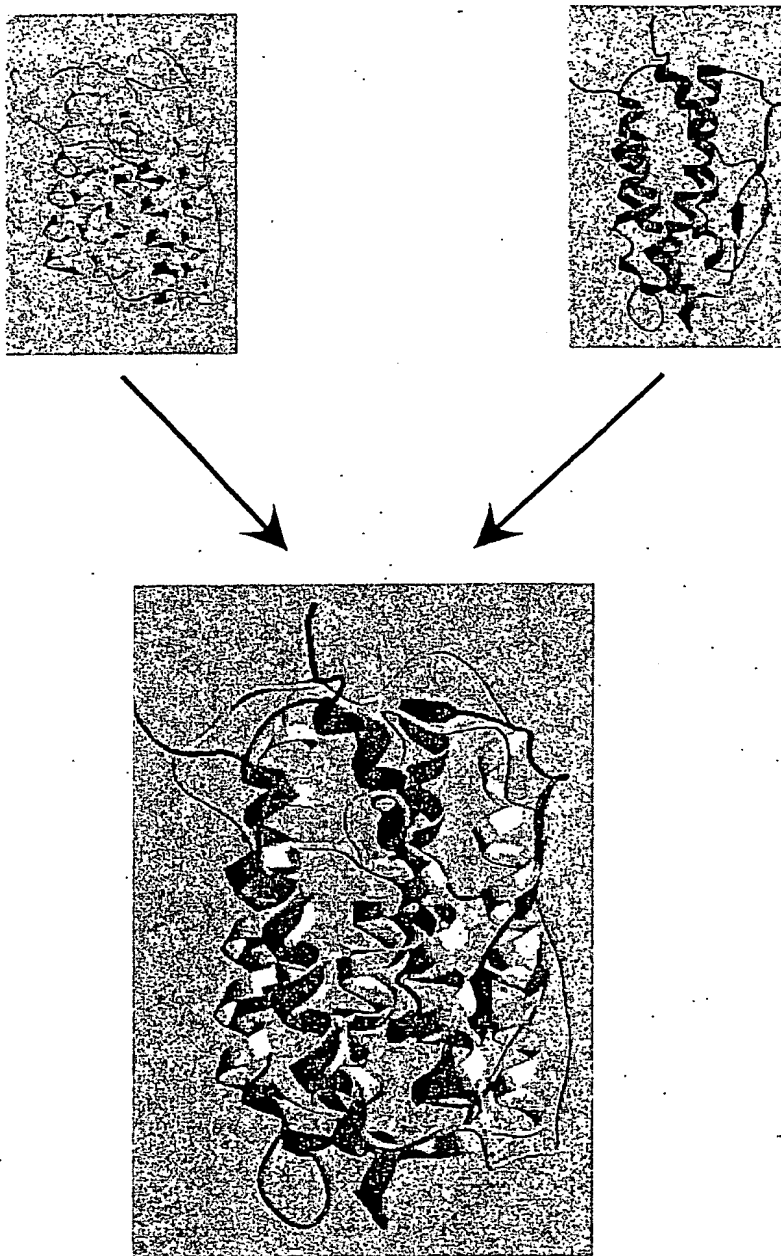


FIG.8C

Structural super-imposition of interferon  $\alpha$ -2b (1RH2)  
and granulocyte-colony stimulating factor (1CD9)

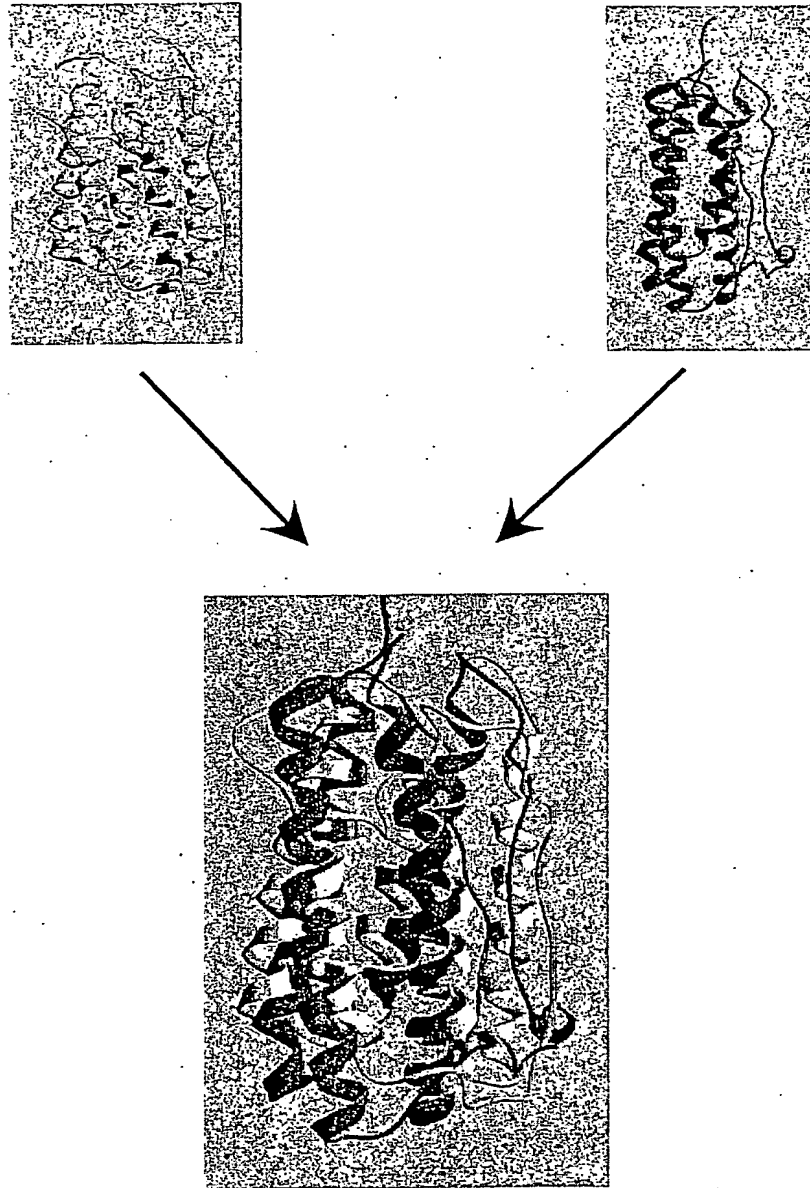


FIG.8D

Cytokine regions susceptible to protease attack identified by structural alignment with Lead mutants

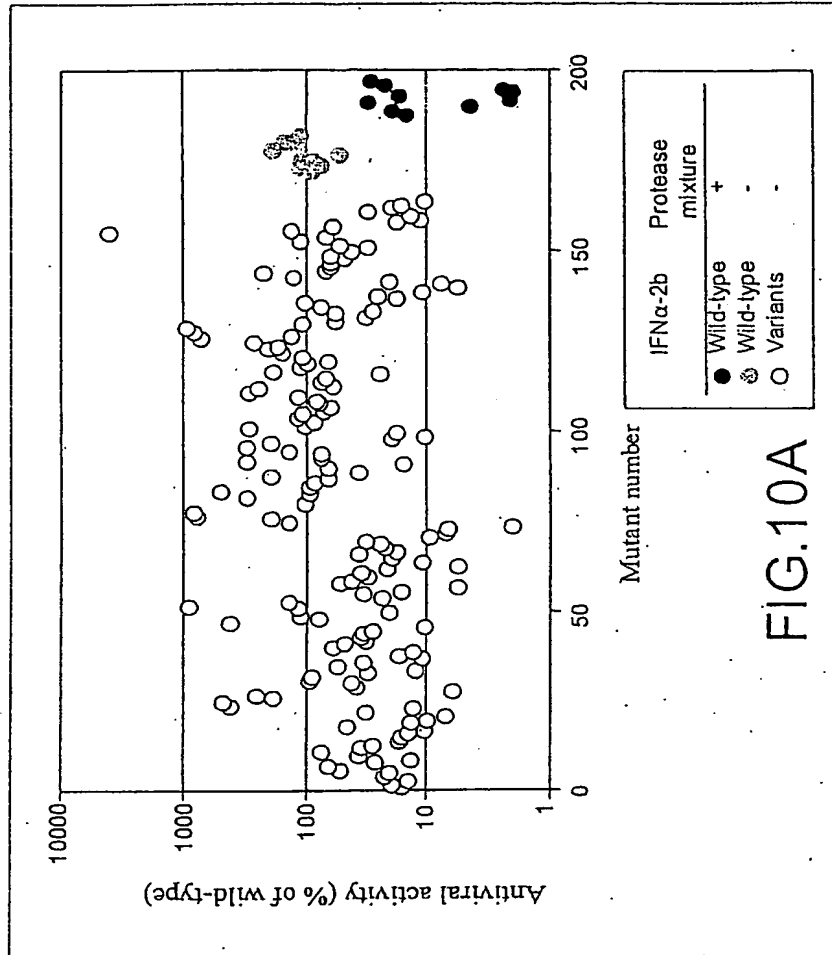
of IFN  $\alpha$ -2b

IFN- $\alpha$ 2b	CDLPQTHSLGSRRTLMLLAQMRKISLFSCLKDRHDFGFPQEEFGNQFQKAETIPVLHEMTQQIFNLFSKDKSSAAWDETLLDKFYTELYQQLNDLEACVTQG VGVTETPLMKEDSILAVRKYFORITLYLKEKKYSFCAMWVVRAEIMRSPSLSSTNLQESLSRSE
Exemplary protein of the interferons/interleukin-10 family	
IFN- $\beta$	MSYNLLGFLQSSNFQCKLLWQLNGRLEYCLKDRMNFDPETIKQLQFQKEDAAATLYEMLQNTFALFRQDSSSTGWNETIVENLLANVYHQINHLKTVLREK LEKEDFTRGKLMSSSLHLKRYIGRIILHYLKAKEYSHCAWTIVRVEILRNFYINRLTGYLRN
Exemplary protein of the short-chain cytokines family	
EPO	APPRLICDSRVLERLYLLEAKEAENITTGCAEHCSLNENITVPDFKVFYAWKRMVQQAQAVEVWQGLALLSEAVLRGQALLVNSSQPWEPLQLHVDKAVSGLRSI TTLLRALGAQKEAISNSDAASAAPLRTITADTFRKLFVRVYSNFLRGKILYTGACRTGDR
Exemplary protein of the long-chain cytokines family	
G-CSF	GPASSLPQSFLKLCLEQVRKIQDGAALQEKLCATYKLCHPRELVLGHSLGIPWAPLSSCPQALQLAGCLSQLHSGFLYQGLLQALEGISPELGPTLDTLQL DVADFATTIWOQMEELGMAPALOPTQCATAPASAFORRAGGVLVASHLQSFLEVSRYRVRHLRAQP

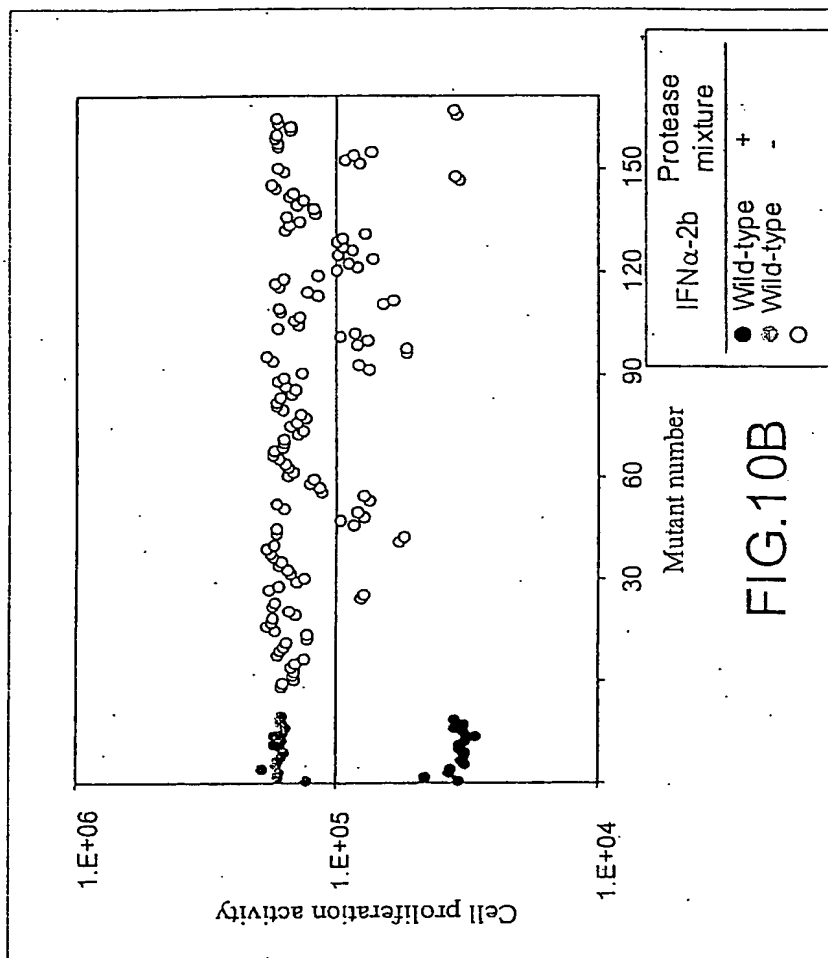
FIG. 9



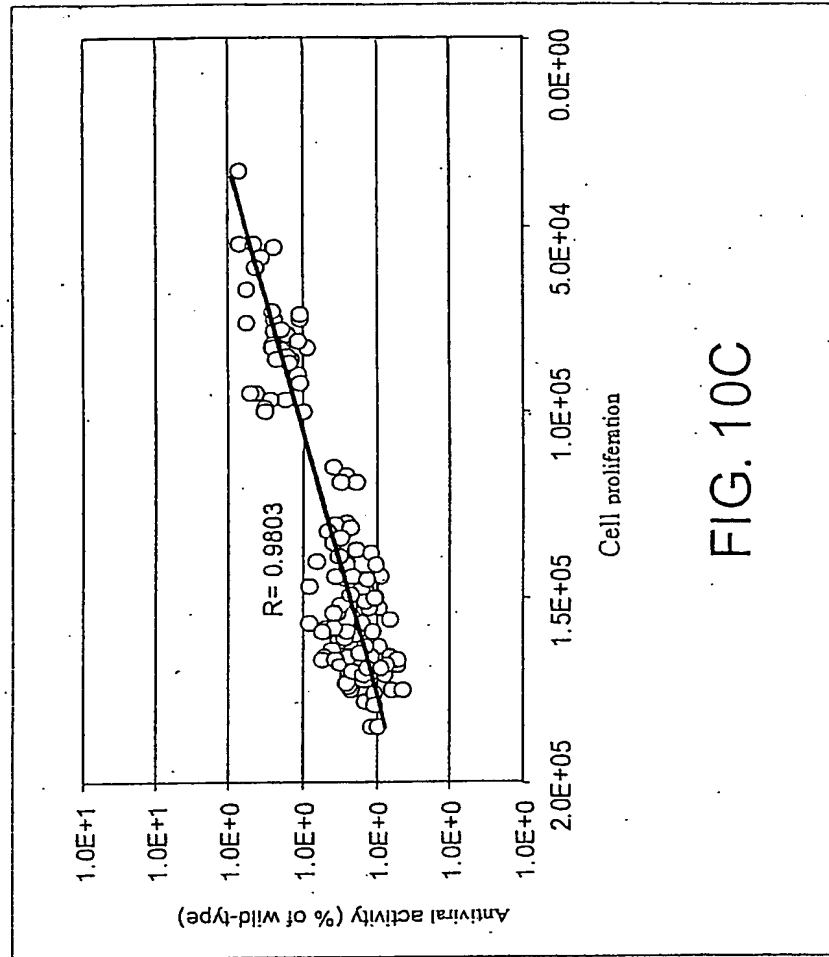
Alanine scanning of interferon  $\alpha$ -2b



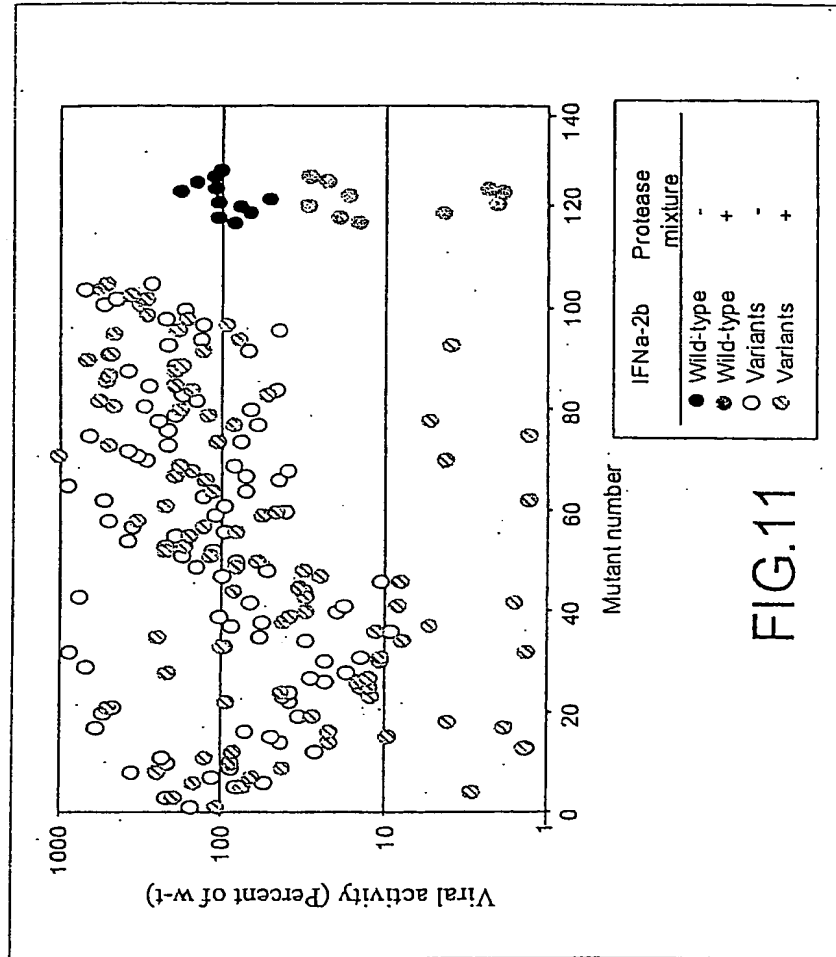
Cell proliferation assay for alanine scanning of interferon  $\alpha$ -2b



Correlation between antiviral and cell proliferation activities for  
alanine scanning of interferon  $\alpha$ -2b



Glycosylation of interferon  $\alpha$ -2b



## Interferon-beta

Protection against proteolysis

Sequence:

MSYNLLGFLQRSSNFQCQKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQQFQKEDAALTI  
 YEMLQNI FAIFRQDSSSTGWNETIVENLLANVYHQINHLKTVLEEKLEKEDFTRGKLMS  
 SLHLKRYYGRI LHYLKAKEYSHCAWTIVRVEILRNFYFINRLTGYLRN

Exposed residues:

-----D--E--KQLQQ-QK-----  
 ----Q--FA--RQD-SS-G-NET-----EKFEDF-R--L--  
 SLH-KR--GR-LH--KAKE-----Y-RN

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
 'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1. D39Q	16. D73Q	31. F111I	46. L130I
2. D39N	17. D73N	32. F111V	47. K134Q
3. E42Q	18. E81Q	33. R113H	48. K134N
4. E42N	19. E81N	34. R113Q	49. K136Q
5. E42H	20. E81H	35. L116V	50. K136N
6. K45Q	21. E107Q	36. L116I	51. E137Q
7. K45N	22. E107N	37. L120V	52. E137N
8. L47V	23. E107H	38. L120I	53. E137H
9. L47I	24. K108Q	39. K123Q	54. Y163H
10. K52Q	25. K108N	40. K123N	55. Y163I
11. K52N	26. E109Q	41. R124H	56. R165H
12. F67I	27. E109N	42. R124Q	57. R165Q
13. F67V	28. E109H	43. R128H	
14. R71H	29. D110Q	44. R128Q	
15. R71Q	30. D110N	45. L130V	

FIG. 12A

## Interferon-gamma

Protection against proteolysis

Sequence:

CYCQDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEESDRKIMQSQIVSFYFKL  
FKNFKDDQSIQKSVETIKEDMNVKFFNSNKKKRDDFEKLTN

Exposed residues:

-----T--L---KN-KEE-----K-  
-KN-KDDQS-----

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1.	L33V	12.	E42H
2.	L33I	13.	K58Q
3.	K37Q	14.	K58N
4.	K37N	15.	K61Q
5.	K40Q	16.	K61N
6.	K40N	17.	K64Q
7.	E41Q	18.	K64N
8.	E41N	19.	D65Q
9.	E41H	20.	D65N
10.	E42Q	21.	D66Q
11.	E42N		

FIG. 12B

## Interleukin-10

Protection against proteolysis

Sequence:

SPGQGTQSENSCTHFPGNLPNMLRLDLRDAFSRVKTFQMKDQLDNLLLLKESLLEDFKGY  
 LGCQALSEMIQFYLEEVMPPQAENQDPDIKAHVNSLGENLKT ,

Exposed residues:

-----KESLLEDFKGY  
 L-----EM-QFY-EEV-PQ-ENQDPD-----K-

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
 'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1. K49Q	18. K57N	35. E75Q
2. K49N	19. Y59H	36. E75N
3. E50Q	20. Y59I	37. E75H
4. E50N	21. L60V	38. P78S
5. E50H	22. L60I	39. P78A
6. L52V	23. E67Q	40. E81Q
7. L52I	24. E67N	41. E81N
8. L53V	25. E67H	42. E81H
9. L53I	26. M68V	43. D84Q
10. E54Q	27. M68I	44. D84N
11. E54N	28. F71I	45. P85S
12. E54H	29. F71V	46. P85A
13. D55Q	30. Y72H	47. D86Q
14. D55N	31. Y72I	48. D86N
15. F56I	32. E74Q	49. K88Q
16. F56V	33. E74N	50. K88N
17. K57Q	34. E74H	

FIG. 12C

## Ciliary neurotrophic factor

Protection against proteolysis

Sequence:

DSADGMPVASTDQWSELTEAERLQENLQAYRTFHVLLARLLEDQQVHFTPTEGDFHQAI  
 HTLLLQVAAFAYQIEELMILLEYKIPRNEADGMPINVGDGGLFEKKLWGLKVLQELSQW  
 TVRSIHDLRFISSHQTGIPA

Exposed residues:

-----VASTDQWSELT-----Q---T-HVL-AR--E--QVH--PTEGD-----  
 -----EYKIPRNE-DGMPINVGDG-L-----  
 -----

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
 'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1. D62Q	16. E92H	31. P135S
2. D62N	17. P100S	32. P135A
3. W64S	18. P100A	33. R136H
4. W64H	19. E102Q	34. R136Q
5. E66Q	20. E102N	35. E138Q
6. E66N	21. E102H	36. E138N
7. E66H	22. D104Q	37. E138H
8. L67V	23. D104N	38. D140Q
9. L67I	24. E131Q	39. D140N
10. L86V	25. E131N	40. P143S
11. L86I	26. E131H	41. P143A
12. R89H	27. Y132H	42. D148Q
13. R89Q	28. Y132I	43. D148N
14. E92Q	29. K133Q	44. L151V
15. E92N	30. K133N	45. L151I

FIG. 12D



## Granulocyte-colony stimulating factor

Protection against proteolysis

Sequence:

VLLGHSLGIPWAPLSSCPSQALQLAGCLSQLHSGFLYQGLLQALEGISPELGPTLDTL  
 QLDVADFATTIWQQMEELGMAPALQPTQGAMPAFASAFQRRAGGVLVASHLQSFLEVS  
 Y  
 RVLRLHLAQP

Exposed residues:

-----W-P-SS-PSQALQ-----S--F-----Q--E--PE-----  
 -----G-APALQPTQ-AM-A-ASAF-----  
 R--RH--QP-

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
 'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1.	W61S	12.	E96N	23.	P135S
2.	W61H	13.	E96H	24.	P135A
3.	P63S	14.	P100S	25.	F147I
4.	P63A	15.	P100A	26.	F147V
5.	P68S	16.	E101Q	27.	R169H
6.	P68A	17.	E101N	28.	R169Q
7.	L72V	18.	E101H	29.	R172H
8.	L72I	19.	P131S	30.	R172Q
9.	F86I	20.	P131A	31.	P177S
10.	F86V	21.	L133V	32.	P177A
11.	E96Q	22.	L133I		

FIG. 12E

## Human growth hormone

Protection against proteolysis

Sequence:

SLCFSESIPTPSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSN  
 VYDLLKDLEEGIQTLMGRLLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRK  
 DMDKVETFLRIVQCRSVEGSCGF

Expose residues:

-----ES-PT-SNREE-----E--QF-RS--AN-L-----  
 -----EDG-PRT-Q--KQTY-KFD-----  
 -----RS-EGSCG-

Proteases: ['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline  
 endopeptidase', 'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1. E56Q	17. F92I	33. K140N
2. E56N	18. F92V	34. Y143H
3. E56H	19. R94H	35. Y143I
4. P59S	20. R94Q	36. K145Q
5. P59A	21. L101V	37. K145N
6. R64H	22. L101I	38. F146I
7. R64Q	23. E129Q	39. F146V
8. E65Q	24. E129N	40. D147Q
9. E65N	25. E129H	41. D147N
10. E65H	26. D130Q	42. R183H
11. E66Q	27. D130N	43. R183Q
12. E66N	28. P133S	44. E186Q
13. E66H	29. P133A	45. E186N
14. E88Q	30. R134H	46. E186H
15. E88N	31. R134Q	
16. E88H	32. K140Q	

FIG. 12F

## Interleukin-12

Protection against proteolysis

Sequence:

DITKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDL  
 KMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVTPQKSSLEEPDFYK  
 TKIKLCILLHAFRIRAVTIDRVMSYLNAS

Exposed residues:

-----KT--VE-----LELTKNES-LNSRETSF-TNGSCLA-RK-----E--  
 KM--VE-KT-N---LM-PKR-----  
 -----R--S--NAS-

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
 'Staphylococcal P.']

Exclusion list:['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1. K56Q	15. E72Q	29. R92H	43. K117Q
2. K56N	16. E72N	30. R92Q	44. K117N
3. E61Q	17. E72H	31. K93Q	45. L124V
4. E61N	18. L75V	32. K93N	46. L124I
5. E61H	19. L75I	33. E107Q	47. M125V
6. L66V	20. R78H	34. E107N	48. M125I
7. L66I	21. R78Q	35. E107H	49. P127S
8. E67Q	22. E79Q	36. K110Q	50. P127A
9. E67N	23. E79N	37. K110N	51. K128Q
10. E67H	24. E79H	38. M111V	52. K128N
11. L68V	25. F82I	39. M111I	53. R129H
12. L68I	26. F82V	40. E115Q	54. R129Q
13. K70Q	27. L89V	41. E115N	55. R189H
14. K70N	28. L89I	42. E115H	56. R189Q

FIG. 12G

## Interleukin-6

Protection against proteolysis

Sequence:

SSKEALAENNLNLPKMAEKDGC FQSGFNEETCLVKIITGLLEFEVYLEYLQNR FESSEE  
 QARAVQMSTKVL IQFLQKKAKNLDAITTPDPTTNASLLTKLQAQNQWLQDMTTHLILRS  
 FKEFLQSSLRALRQM

Exposed residues:

-----PKMAEK---FQSGF-----T---E---E---QNR-ES-E-  
 -----DA-TTPDPTT-AS--TK-QAQNQW-----  
 -----R--RQM

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
 'Staphylococcal P.']

Exclusion list: ['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1. P64S	16. E92Q	31. D133N
2. P64A	17. E92N	32. P138S
3. K65Q	18. E92H	33. P138A
4. K65N	19. E98Q	34. D139Q
5. M66V	20. E98N	35. D139N
6. M66I	21. E98H	36. P140S
7. E68Q	22. R103H	37. P140A
8. E68N	23. R103Q	38. K149Q
9. E68H	24. E105Q	39. K149N
10. K69Q	25. E105N	40. W156S
11. K69N	26. E105H	41. W156H
12. F73I	27. E108Q	42. R178H
13. F73V	28. E108N	43. R178Q
14. F77I	29. E108H	44. R181H
15. F77V	30. D133Q	45. R181Q

FIG. 12H

## Leptin

Protection against proteolysis

Sequence:

VPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGLDFIPGLHPILTLKMDQTLA  
VYQQILTSMPSRNVIQISNDLENLRDLLHVLAFSKSCHLPWASGLETLDLGGVLEASG  
YSTEVVALSRLQGSLLQDMLWQLDLSPGC

Exposed residues:

-----P--H-IL-----  
-----SCH-PW-SGLETLDL--GV-----  
-----DLS-GC

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1.	P43S	12.	E105N
2.	P43A	13.	E105H
3.	L49V	14.	L107V
4.	L49I	15.	L107I
5.	P99S	16.	D108Q
6.	P99A	17.	D108N
7.	W100S	18.	D141Q
8.	W100H	19.	D141N
9.	L104V	20.	L142V
10.	L104I	21.	L142I
11.	E105Q		

FIG. 12I

## Leukemia inhibitory factor

Protection against proteolysis

Sequence:

PFPNNLDKLCGPNVTDFPPFHANGTEKAKLVELYRIVVYLGTSLGNITRDQKILNPSAL  
 SLHSKLNATADILRGLLSNVLCRLCSKYHVGHVDTYGPDTSGKDVFQKKKLGCGLLGK  
 YKQIIAVLAQAF

Exposed residues:

-----PFHAN-T-----R-----T-----R--KIL-PS-  
 LS-----YH-GHVDVTYGPD-SGKDVF-----  
 -----Q---

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
 'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1. P69S	12. L104I	23. P148S
2. P69A	13. P106S	24. P148A
3. F70I	14. P106A	25. D149Q
4. F70V	15. L109V	26. D149N
5. R85H	16. L109I	27. K153Q
6. R85Q	17. Y137H	28. K153N
7. R99H	18. Y137I	29. D154Q
8. R99Q	19. D143Q	30. D154N
9. K102Q	20. D143N	31. F156I
10. K102N	21. Y146H	32. F156V
11. L104V	22. Y146I	

FIG. 12J

## Oncostatin M

Protection against proteolysis

Sequence:

ERPGAFPSEETLRGLGRRGFLQTLNATLGCVLHRLADLEQRLPKAQDLERSGLNIEDLE  
KLQMARPNILGLRNNIYCMAQLLDNSDTAEPTKAGRGASQP

Exposed residues:

-----SEET-RGLG-----NA---C--HR-AD-EQR--KAQD-ERSGLNIE---

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1.	E59Q	12.	R84Q	23.	D97N
2.	E59N	13.	D87Q	24.	E99Q
3.	E59H	14.	D87N	25.	E99N
4.	E60Q	15.	E89Q	26.	E99H
5.	E60N	16.	E89N	27.	R100H
6.	E60H	17.	E89H	28.	R100Q
7.	R63H	18.	R91H	29.	L103V
8.	R63Q	19.	R91Q	30.	L103I
9.	L65V	20.	K94Q	31.	E106Q
10.	L65I	21.	K94N	32.	E106N
11.	R84H	22.	D97Q	33.	E106H

FIG. 12K

## Erythropoietin

Protection against proteolysis

Sequence:

APPRLICDSRVLERYLLEAKEAENITTGCAEHCSLNENITVPDTKVNIFYAWKRMEVGQQ  
AVEVWQGLALLSEAVLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTLRLALGAQKEA  
ISPPDAASAAPLRTITADTFRKLFRVYSNFLRGKCLKLYTGEACRTGDR

Exposed residues:

-----N-T--DTKVNIFYA-KR-EV---  
-----A--SE--LR-QA--VNSSQ-----  
ISPPDA-SAAPLR-IT-----RTGDR

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1. D43Q	14. E55N	27. L130V
2. D43N	15. E55H	28. L130I
3. K45Q	16. E72Q	29. R131H
4. K45N	17. E72N	30. R131Q
5. F48I	18. E72H	31. R162H
6. F48V	19. L75V	32. R162Q
7. Y49H	20. L75I	33. D165Q
8. Y49I	21. R76H	34. D165N
9. K52Q	22. R76Q	35. P121S
10. K52N	23. D123Q	36. P121A
11. R53H	24. D123N	37. P122S
12. R53Q	25. P129S	38. P122A
13. E55Q	26. P129A	

FIG. 12L



## Flt3 ligand

Protection against proteolysis

Sequence:

TQDCSFQHSPISSDFAVKIRELSDYLLQDYPVTVASNLQDEELCGGLWRLVLAQRWMER  
LKTVAGSKMQGLLERVNTTEIHFVTKCAFQPPPSCLRFVQTN

Exposed residues:

TQD-----T--S--QD-EL-----R--ER  
-KTV-G-----A-QPPPSCL-RFV---

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1.	D3Q	15.	R59H
2.	D3N	16.	R59Q
3.	D40Q	17.	K61Q
4.	D40N	18.	K61N
5.	E42Q	19.	P89S
6.	E42N	20.	P89A
7.	E42H	21.	P90S
8.	L43V	22.	P90A
9.	L43I	23.	P91S
10.	R55H	24.	P91A
11.	R55Q	25.	R95H
12.	E58Q	26.	R95Q
13.	E58N	27.	F96I
14.	E58H	28.	F96V

FIG. 12M

## Granulocyte-macrophage colony-stimulating factor

Protection against proteolysis

Sequence:

APARSPSPSTQPWEHVNAIQEARRLLNLSRDTAAEMNETVEVISEMFDLQEPTCLQTRL  
 ELYKQGLRGS�TKLKGPLTMMASHYKQHCPTPETSCATQIITFESFKENLKDFLLVIP  
 FDCWEPVQE

Exposed residues:

-----ET-E--SEM-DLQE-----  
 E--KQ--R-----PETSCATQI-T-----  
 FD--EP---

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
 'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1. E38Q	14. L49V	27. P92A
2. E38N	15. L49I	28. E93Q
3. E38H	16. E51Q	29. E93N
4. E41Q	17. E51N	30. E93H
5. E41N	18. E51H	31. F119I
6. E41H	19. E60Q	32. F119V
7. E45Q	20. E60N	33. D120Q
8. E45N	21. E60H	34. D120N
9. E45H	22. K63Q	35. E123Q
10. M46V	23. K63N	36. E123N
11. M46I	24. R67H	37. E123H
12. D48Q	25. R67Q	38. P124S
13. D48N	26. P92S	39. P124A

FIG. 12N

## Interleukin-13

Protection against proteolysis

Sequence:

GPVPPSTALRELIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAALESLINVSGCSAI  
EKTQRMLSGFCPHKVSAGQFSSLHVRDTKIEVAQFVKDLLLHLKKLFREGRFN

Exposed residues:

-----M-WS-NLTAG-----E--INVSG-----  
-----AGQFSSLHVRDTK-----REGRFN

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
'Staphylococcal P.']

Exclusion list: ['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1. M32V	11. F79V	21. R107Q
2. M32I	12. L82V	22. E108Q
3. W34S	13. L82I	23. E108N
4. W34H	14. R85H	24. E108H
5. L38V	15. R85Q	25. R110H
6. L38I	16. D86Q	26. R110Q
7. E48Q	17. D86N	27. F111I
8. E48N	18. K88Q	28. F111V
9. E48H	19. K88N	
10. F79I	20. R107H	

FIG. 120

## Interleukin-2

Protection against proteolysis

Sequence:

APTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L T F K F Y M P K K A T E L K H L Q C L  
 E E L K P L E E V L N L A Q S K N F H L R P R D L I S N I N V I V L E L K G S E T T F M C E Y A D E T A T I V E F L  
 N R W I T F C Q S I I S T L T

Exposed residues:

-----K-Y--KKATEL---Q--  
 EE--KP-EE--NL-----ETTFM-EYADET-T-----  
 -----STLT

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
 'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1. K43Q	13. L53I	25. E68Q	37. E106Q
2. K43N	14. E60Q	26. E68N	38. E106N
3. Y45H	15. E60N	27. E68H	39. E106H
4. Y45I	16. E60H	28. L72V	40. Y107H
5. K48Q	17. E61Q	29. L72I	41. Y107I
6. K48N	18. E61N	30. E100Q	42. D109Q
7. K49Q	19. E61H	31. E100N	43. D109N
8. K49N	20. P65S	32. E100H	44. E110Q
9. E52Q	21. P65A	33. F103I	45. E110N
10. E52N	22. E67Q	34. F103V	46. E110H
11. E52H	23. E67N	35. M104V	47. L132V
12. L53V	24. E67H	36. M104I	48. L132I

FIG. 12P

## Interleukin-3

Protection against proteolysis

Sequence:

APMTQTTPPKTSWVNCSNMIDEIITHLKQPPLPLLDNFNNLNGEDQDILMENNLRPNLE  
AFNRAVKSLQNASAIESILKNLLPCLPLATAAPTRHPIHIKGDWNEFRKLTFFYLKTL  
ENAQAQQTTLSLAIF

Exposed residues:

-----F-N-NGE-QD-----E  
--R--KS-Q-----HP-H-KD-D-----  
-----

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1.	F37I	12.	R63Q
2.	F37V	13.	K66Q
3.	E43Q	14.	K66N
4.	E43N	15.	P96S
5.	E43H	16.	P96A
6.	D46Q	17.	K100Q
7.	D46N	18.	K100N
8.	E59Q	19.	D101Q
9.	E59N	20.	D101N
10.	E59H	21.	D103Q
11.	R63H	22.	D103N

FIG. 12Q

## Interleukin-4

Protection against proteolysis

Sequence:

HKCDITLQEI I KTLNSLTEQKTLCTELTVTDIFAASKNTTEKETFCRAATVLRQFYSHH  
EKDTRCLGATAQQFHRHKQLIRFLKRLDRNLWGLAGLNSCPVKEANQSTLENFLERLKT  
IMREKYSKCSS

Exposed residues:

-----E-T-----AASKNTT-----RQ--SH-  
EK-TR-L-----SCPVKEANQ-----  
-----KCSS

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

- |          |           |
|----------|-----------|
| 1. E26Q  | 14. R64Q  |
| 2. E26N  | 15. L66V  |
| 3. E26H  | 16. L66I  |
| 4. K37Q  | 17. P100S |
| 5. K37N  | 18. P100A |
| 6. R53H  | 19. K102Q |
| 7. R53Q  | 20. K102N |
| 8. E60Q  | 21. E103Q |
| 9. E60N  | 22. E103N |
| 10. E60H | 23. E103H |
| 11. K61Q | 24. K126Q |
| 12. K61N | 25. K126N |
| 13. R64H |           |

FIG. 12R

## Interleukin-5

Protection against proteolysis

Sequence:

IPTEIPTSALVKETLALLSTHRTLLIANETLRIPVPVHKNHQLCTEEIFQGIGTLESQT  
VQGGTVERLFFKNLSLIKYYIDGQKKKCGEERRRVNQFLDYLQEFGLGVMNTEWIIES

Exposed residues:

-----R-P--V-K-----EE--Q--GT-ESQ-  
-----KK-GEER-----E-----NTEW----

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1. R32H	13. E56Q	25. E89H
2. R32Q	14. E56N	26. R90H
3. P34S	15. E56H	27. R90Q
4. P34A	16. K84Q	28. E102Q
5. K39Q	17. K84N	29. E102N
6. K39N	18. K85Q	30. E102H
7. E46Q	19. K85N	31. E110Q
8. E46N	20. E88Q	32. E110N
9. E46H	21. E88N	33. E110H
10. E47Q	22. E88H	34. W111S
11. E47N	23. E89Q	35. W111H
12. E47H	24. E89N	

FIG. 12S

## Stem cell factor

Protection against proteolysis

Sequence:

EGICRNRVTNNVKDVT'KL'VANLPKDYMITLK'YVPGMDVLP'SHCW'ISEMVVQLSDSLTDL  
 LDKFSNISEGLSNYSIIDKLVNI'VDDLVE'CVKENSSKDLKKSFKSPEPRLFTPEEFFRI  
 FNRSIDAFKDFVVA'SETSDCVVS

Exposed residues:

-----M-T-K--P--DV-----V---D--TD-  
 -DKFSN-----SK-LKKSFKS-EPRL-----  
 -----ASETSDCVVS

Proteases:

['Trypsin', 'Endoproteinase Asp-N', 'Chymotrypsin', 'Proline endopeptidase',  
 'Staphylococcal P.']

Exclusion list:

['B', 'Z', 'X', '\*', 'K', 'R', 'D', 'F', 'W', 'Y', 'M', 'L', 'P', 'E']

Substitutions:

1.	M27V	16.	K62N	31.	E106Q
2.	M27I	17.	F63I	32.	E106N
3.	K31Q	18.	F63V	33.	E106H
4.	K31N	19.	K96Q	34.	P107S
5.	P34S	20.	K96N	35.	P107A
6.	P34A	21.	L98V	36.	R108H
7.	D37Q	22.	L98I	37.	R108Q
8.	D37N	23.	K99Q	38.	L109V
9.	D54Q	24.	K99N	39.	L109I
10.	D54N	25.	K100Q	40.	E134Q
11.	D58Q	26.	K100N	41.	E134N
12.	D58N	27.	F102I	42.	E134H
13.	D61Q	28.	F102V	43.	D137Q
14.	D61N	29.	K103Q	44.	D137N
15.	K62Q	30.	K103N		

FIG. 12T



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
18 March 2004 (18.03.2004)

PCT

(10) International Publication Number  
**WO 2004/022593 A3**

(51) International Patent Classification<sup>7</sup>: C12N 15/19,  
15/20, 15/24, G06F 19/00, C07K 14/52, 14/53, 14/535,  
14/54, 14/555, 14/61, 14/505, 14/475, 14/575, C12Q 1/68,  
A61K 38/19

(21) International Application Number:  
PCT/IB2003/004347

(22) International Filing Date:  
8 September 2003 (08.09.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/409,898 9 September 2002 (09.09.2002) US  
60/457,135 21 March 2003 (21.03.2003) US

(71) Applicant (for all designated States except US): NAU-  
TILUS BIOTECH [FR/FR]; 1 rue Pierre Fontaine,  
F-91000 EVRY (FR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): GANTIER, René  
[FR/FR]; 2 rue de Dublin, La Clef de Saint-Pierre, F-78990  
ELANCOURT (FR). GUYON, Thierry [FR/FR]; 8 rési-  
dence les Terres Rouges, F-91120 PALAISEAU (FR).  
VEGA, Manuel [IT/FR]; 49 rue Félix Faure, F-91270 VI-  
GNEUX-SUR-SEINE (FR). DRITTANTI, Lila [IT/FR];  
49 rue Félix Faure, F-91270 VIGNEUX-SUR-SEINE  
(FR).

(74) Agent: CABINET ORES; 36 rue de St Pétersbourg,  
F-75008 PARIS (FR).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC,  
SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA,  
UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,  
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO,  
SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM,  
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

- with international search report
- before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments
- with sequence listing part of description published sepa-  
rately in electronic form and available upon request from  
the International Bureau

(88) Date of publication of the international search report:  
15 July 2004

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: RATIONAL EVOLUTION OF CYTOKINES FOR HIGHER STABILITY, THE CYTOKINES AND ENCODING NU-  
CLEIC ACID MOLECULES

(57) Abstract: Processes and systems for the high throughput directed evolution of peptides and proteins, particularly cytokines that  
act in complex biological settings, are provided. Also provided is a rational method for generating protein variants and the resulting  
variants.

WO 2004/022593 A3

## INTERNATIONAL SEARCH REPORT

PCT/IB 03/04347

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7	C12N15/19	C12N15/20	C12N15/24	G06F19/00	C07K14/52
	C07K14/53	C07K14/535	C07K14/54	C07K14/555	C07K14/61
	C07K14/505	C07K14/475	C07K14/575	C12Q1/68	A61K38/19

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N G06F C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EP0-Internal, BIOSIS, Sequence Search, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	-/--	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

16 January 2004

Date of mailing of the international search report

14 05. 2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Andres, S

## INTERNATIONAL SEARCH REPORT

PCT/IB 03/04347

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 240 224 A (INTERFERON SCIENCES INC) 7 October 1987 (1987-10-07)	1-5,8, 11,24, 26-28, 31-45, 139-141, 279,280, 307-309, 312-317, 319,332, 335-337, 340,341, 343-346, 348
Y	the whole document	10-22, 25-30, 139-141, 306-315, 325, 334-341
X	----- WO 01/25438 A (MAXYGEN INC ; CHEN TEDDY (US); HEINRICHS VOLKER (US); PATTEN PHILLIP A) 12 April 2001 (2001-04-12) page 3 - page 6 page 64 page 79 - page 81 page 90 - page 100	1,279, 280
Y		10-12, 14, 25-30, 306-315, 334-341
X	----- PIEHLER JACOB ET AL: "New structural and functional aspects of the type I interferon-receptor interaction revealed by comprehensive mutational analysis of the binding interface" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, no. 51, 22 December 2000 (2000-12-22), pages 40425-40433, XP002267030 ISSN: 0021-9258 cited in the application page 40428; table I	1,279, 280
Y	figures 3,4	10-22, 139-141, 325
A	----- GIBRAT JEAN-FRANCOIS ET AL: "Surprising similarities in structure comparison" CURRENT OPINION IN STRUCTURAL BIOLOGY, vol. 6, no. 3, 1996, pages 377-385, XP002267031 ISSN: 0959-440X cited in the application ----- -/--	

## INTERNATIONAL SEARCH REPORT

PCT/IB 03/04347

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HOLM L ET AL: "MAPPING THE PROTEIN UNIVERSE" SCIENCE, vol. 273, 2 August 1996 (1996-08-02), pages 595-602, XP002916511 ISSN: 0036-8075 cited in the application -----	
A	WO 01/61344 A (VOIGT CHRISTOPHER ; MAYO STEPHEN L (US); WANG ZHEN GANG (US); ARNOLD F) 23 August 2001 (2001-08-23) -----	

# INTERNATIONAL SEARCH REPORT

PCT/IB 03/04347

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 31-35, 312-315, 340, 341, 343 and 344 (as far as in vivo methods are concerned) and claims 36, 316 and 345 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see FURTHER INFORMATION sheet, invention 1.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 31-35,312-315,340,341,343 and 344 (as far as in vivo methods are concerned) and claims 36,316 and 345 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention 1.: Claims 1-5,8,10-45,139-141,279,280,  
306-319,325,332,334-341,343-349 (all partially and as far as  
applicable)

A modified interferon alpha-2b characterised by SEQ ID 2;  
nucleic acids encoding it and cells expressing it;  
compositions comprising the IFN or the nucleic acids  
encoding it.

---

Inventions 2. to 181.: Claims 1-45,139-141,279,280,  
306-319,325,332,334-341,343-349 (all partially and as far as  
applicable)

As for invention 1., but concerning respectively SEQ IDs  
3-181 and 1303.

---

Invention 182.: Claims 1-13,16-21,24-46,279,280,  
306-322,325,332,334-341,343-349 (all partially and as far as  
applicable) and claims 47,48,142-144

As for Invention 1., but concerning modified IFN alpha-2a.

---

Invention 183.: Claims 1-7,10,14-21,24-46,279,280,  
306-322,325,332,334-341,343-349 (all partially and as far as  
applicable) and claims 51,52,148-150

As for invention 182., but concerning modified IFN alpha-2c.

---

Invention 184.: Claims 1-5,10,14-21,24-46,279,280,  
306-319,325,332,334-341,343-349 (all partially and as far as  
applicable) and claims 73,74,181-183

As for invention 182., but concerning consensus IFN alpha.

---

Invention 185.: Claims 1-4,21,24-32,34-40,42-46,279,280,  
306-319,325,332,334-341,343-349 (all partially and as far as  
applicable) and claims 49,50,145-147

As for invention 182., but concerning IFN alpha-c.

---

Inventions 186. to 195.: Claims 1-4,21,24-32,34-40,42-46,53-72,  
151-180,279,280,306-319,325,332,334-341,343-349 (all  
partially and as far as applicable)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

As for invention 185., but concerning respectively all other IFN alphas disclosed in claim 46.

---

Invention 196.: Claims 1-4,21,24-32,34-40,75-77,184-186, 279,280,306-319,323-375 (all partially and as far as applicable)

As for invention 1., but concerning IFN beta.

---

Inventions 197. and 198.: Claims 1-4,21,24-32,34-40,78-81,187-192, 279,280,306-319,323-375 (all partially and as far as applicable)

As for invention 196., but concerning respectively IFN beta-1 and IFN beta-2a.

---

Invention 199.: Claims 1-4,21,24-32,34-40,279,280, 306-319,325,332,334-341,343-349 (all partially and as far as applicable) and claims 82-84,193-195

As for invention 1., but concerning IFN gamma.

---

Inventions 200. to 217.: Claims 1-3,21,24-32,34-40,85-138,196-249,279,280,306-319,325, 332,334-341,3

3-349 (all partially and as far as applicable)

As for invention 1., but concerning respectively IL-10, EP0, GM-CSF, Flt3-ligand, IL-2, IL-3, SCF, IL-4, IL-5, IL-13, G-CSF, leptin, CNTF, LIF, oncostatin M, IL-12, hGH and IL-6.

---

Invention 218.: Claims 250-278 and 281-305

Methods for generating a protein having a predetermined property or a pre-selected altered phenotype.

---



## INTERNATIONAL SEARCH REPORT

PCT/IB 03/04347

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0240224	A	07-10-1987	CN 87102497 A	11-11-1987
			DK 161487 A	01-10-1987
			EP 0240224 A2	07-10-1987
			JP 62296892 A	24-12-1987
-----				
WO 0125438	A	12-04-2001	AU 8001300 A	10-05-2001
			CA 2385045 A1	12-04-2001
			CN 1451045 T	22-10-2003
			EP 1238082 A2	11-09-2002
			JP 2003511031 T	25-03-2003
			WO 0125438 A2	12-04-2001
			US 2004002474 A1	01-01-2004
-----				
WO 0161344	A	23-08-2001	AU 3839701 A	27-08-2001
			WO 0161344 A1	23-08-2001
			US 2001051855 A1	13-12-2001
-----				

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**